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"A role for the transcriptional repressor ICER in the molecular pathogenesis of type 2 diabetes"

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A role for the transcriptional repressor ICER in the molecular pathogenesis of type 2 diabetes

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Résumé

Rôle du répresseur transcriptionnel ICER dans la pathogenèse moléculaire du diabète de type 2

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Le diabète de type 2 (DT2) est une maladie métabolique qui affecte plus de 200 millions de personnes dans le monde. La progression de cette affection atteint aujourd'hui des proportions épidémiques imputables à l'augmentation rapide dans les fréquences du surpoids, de l'obésité et de la sédentarité. La pathogenèse du DT2 se caractérise par une diminution de l'action de l'insuline sur ses tissus cibles – un processus nommé insulino-résistance – ainsi qu'une dysfonction des cellules β pancréatiques sécrétrices d'insuline. Cette dernière détérioration se définit par une réduction de la capacité de synthèse et de sécrétion de l'insuline et mène finalement à une perte de la masse de cellules β par apoptose. Des facteurs environnementaux fréquemment associés au DT2, tels l'élévation chronique des taux plasmatiques de glucose et d'acides gras libres, les cytokines pro-inflammatoires et les lipoprotéines de faible densité (LDL) oxydées, contribuent à la perte de fonction des cellules β pancréatiques.

Dans cette étude, nous avons démontré que le facteur de transcription « Inducible Cyclic AMP Early Repressor » (ICER) participe à la progression de la dysfonction des cellules β pancréatiques et au développement de l'insulino-résistance. Son expression étant gouvernée par un promoteur alternatif, la protéine d'ICER représente un produit tronqué de la famille des « Cyclic AMP Response Element Modulator » (CREM), sans domaine de transactivation. Par conséquent, le facteur ICER agit comme un répresseur passif qui réduit l'expression des gènes contrôlés par la voie de l'AMP cyclique et des « Cyclic AMP Response Element Binding protein » (CREB).

Dans les cellules sécrétrices d'insuline, l'accumulation de radicaux d'oxygène libres, soutenue par les facteurs environnementaux et notamment les LDL oxydées – un processus appelé stress oxydatif – induit de manière ininterrompue le facteur de transcription ICER. Ainsi activé, ce répresseur transcriptionnel altère la capacité sécrétoire des cellules β en bloquant l'expression de gènes clés de la machinerie d'exocytose. En outre, le facteur ICER favorise l'activation de la cascade de signalisation « c-Jun N-terminal Kinase » (JNK) en réduisant l'expression de la protéine « Islet Brain 1 » (IB1), altérant ainsi les fonctions de biosynthèse de l'insuline et de survie des cellules β pancréatiques.

Dans le tissu adipeux des souris et des sujets humains souffrant d'obésité, le facteur de transcription ICER contribue à l'altération de la réponse à l'insuline. La disparition de la protéine ICER dans ces tissus entraîne une activation persistante de la voie de signalisation des CREB et une induction du facteur de transcription « Activating Transcription Factor 3 » (ATF3). A son tour, le répresseur ATF3 inhibe l'expression du transporteur de glucose GLUT4 et du peptide adipocytaire insulino-sensibilisateur adiponectine, contribuant ainsi à la diminution de l'action de l'insuline en conditions d'obésité.

En conclusion, à la lumière de ces résultats, le répresseur transcriptionnel ICER apparaît comme un facteur important dans la pathogenèse du DT2, en participant à la perte de fonction des cellules β pancréatiques et à l'aggravation de l'insulino-résistance. Par conséquent, l'étude des mécanismes moléculaires responsables de l'altération des niveaux du facteur ICER pourrait permettre le développement de nouvelles stratégies de traitement du DT2.

Abstract

A role for the transcriptional repressor ICER in the molecular pathogenesis of type 2 diabetes

Dimitri Favre, Service de médecine interne, Centre Hospitalier Universitaire Vaudois

Type 2 diabetes (T2D) is a metabolic disease which affects more than 200 millions people worldwide. The progression of this affection reaches nowadays epidemic proportions, owing to the constant augmentation in the frequency of overweight, obesity and sedentary. The pathogenesis of T2D is characterized by reduction in the action of insulin on its target tissues – an alteration referred as insulin resistance – and pancreatic β-cell dysfunction. This latter deterioration is defined by impairment in insulin biosynthesis and secretion, and a loss of β-cell mass by apoptosis. Environmental factors related to T2D, such as chronic elevation in glucose and free fatty acids levels, inflammatory cytokines and pro-atherogenic oxidized low-density lipoproteins (LDL), contribute to the loss of pancreatic β-cell function.

In this study, we have demonstrated that the transcription factor Inducible Cyclic AMP Early Repressor (ICER) participates to the progression of both β-cell dysfunction and insulin resistance. The expression of this factor is driven by an alternative promoter and ICER protein represents therefore a truncated product of the Cyclic AMP Response Element Modulator (CREM) family which lacks transactivation domain. Consequently, the transcription factor ICER acts as a passive repressor which reduces expression of genes controlled by the cyclic AMP and Cyclic AMP Response Element Binding protein (CREB) pathway.

In insulin-secreting cells, the accumulation of reactive oxygen species caused by environmental factors and notably oxidized LDL – a process known as oxidative stress – induces the transcription factor ICER. This transcriptional repressor hampers the secretory capacity of β-cells by silencing key genes of the exocytotic machinery. In addition, the factor ICER reduces the expression of the scaffold protein Islet Brain 1 (IB1), thereby favouring the activation of the c-Jun N-terminal Kinase (JNK) pathway. This triggering alters in turn insulin biosynthesis and survival capacities of pancreatic β-cells.

In the adipose tissue of mice and human subjects suffering from obesity, the transcription factor ICER contributes to the alteration in insulin action. The loss in ICER protein in these tissues induces a constant activation of the CREB pathway and the subsequent expression of the Activating Transcription Factor 3 (ATF3). In turn, this repressor reduces the transcript levels of the glucose transporter GLUT4 and the insulin-sensitizer peptide adiponectin, thereby contributing to the diminution in insulin action.

In conclusion, these data shed light on the important role of the transcriptional repressor ICER in the pathogenesis of T2D, which contributes to both alteration in β-cell function and aggravation of insulin resistance. Consequently, a better understanding of the molecular mechanisms responsible for the alterations in ICER levels is required and could lead to develop new therapeutic strategies for the treatment of T2D.

Résumé didactique

Rôle du répresseur transcriptionnel ICER dans la pathogenèse moléculaire du diabète de type 2

Dimitri Favre, Service de médecine interne, Centre Hospitalier Universitaire Vaudois

L'énergie nécessaire au bon fonctionnement de l'organisme est fournie par l'alimentation, notamment sous forme de sucres (glucides). Ceux-ci sont dégradés en glucose, lequel sera distribué aux différents organes par la circulation sanguine. Après un repas, le niveau de glucose sanguin, nommé glycémie, s'élève et favorise la sécrétion d'une hormone appelée insuline par les cellules β du pancréas. L'insuline permet, à son tour, aux organes, tels le foie, les muscles et le tissu adipeux de capter et d'utiliser le glucose ; la glycémie retrouve ainsi son niveau basal.

Le diabète de type 2 (DT2) est une maladie métabolique qui affecte plus de 200 millions de personnes dans le monde. Le développement de cette affection est causée par deux processus pathologiques. D'une part, les quantités d'insuline secrétée par les cellules β pancréatiques, ainsi que la survie de ces cellules sont réduites, un phénomène connu sous le nom de dysfonction des cellules β. D'autre part, la sensibilité des tissus à l'insuline se trouve diminuée. Cette dernière altération, l'insulino-résistance, empêche le transport et l'utilisation du glucose par les tissus et mène à une accumulation de ce sucre dans le sang. Cette stagnation de glucose dans le compartiment sanguin est appelée hyperglycémie et favorise l'apparition des complications secondaires du diabète, telles que les maladies cardiovasculaires, l'insuffisance rénale, la cécité et la perte de sensibilité des extremités.

Dans cette étude, nous avons démontré que le facteur ICER qui contrôle spécifiquement l'expression de certains gènes, contribue non seulement à la dysfonction des cellules β, mais aussi au développement de l'insulino-résistance. En effet, dans les cellules β pancréatiques en conditions diabétiques, l'activation du facteur ICER altère la capacité de synthèse et de sécrétion d'insuline et réduit la survie ces cellules.

Dans le tissu adipeux des souris et des sujets humains souffrant d'obésité, le facteur ICER contribue à la perte de sensibilité à l'insuline. La disparition d'ICER altère l'expression de la protéine qui capte le glucose, le transoprteur GLUT4, et l'hormone adipocytaire favorisant la sensibilité à l'insuline, nommée adiponectine. Ainsi, la perte d'ICER participe à la réduction de la captation de glucose par le tissue adipeux et au développement de l'insulino-résistance au cours de l'obésité.

En conclusion, à la lumière de ces résultats, le facteur ICER apparaît comme un contributeur important à la progression du DT2, en soutenant la dysfonction des cellules β pancréatiques et l'aggravation de l'insulino-résistance. Par conséquent, l'étude des mécanismes responsables de la dérégulation du facteur ICER pourraît permettre le développement de nouvelles stratégies de traitement du DT2.

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I Introduction

I Introduction

A Glucose homeostasis

Glucose represents a major source of energy for the body and is an indispensable fuel for neuronal tissues. Consequently, the maintenance of blood glucose levels in a narrow range – a process referred as glucose homeostasis – is of primordial importance and relies on a complex interaction between neurological and humoral pathways, where insulin possesses a dominant position. After a meal ingestion, blood glucose level raises and triggers insulin secretion from pancreatic β-cells. The elevation in insulinemia favors glucose uptake and utilization predominantly by the muscle, liver and adipose tissue, thereby restoring basal glycemia.

A.1 Insulin secretion

A.1.1 β-cell of the islet of Langerhans

The endocrine pancreas consists of small group of cells scattered among the exocrine part of the organ. The endocrine unit is known as the pancreatic islets, or the *islets of Langerhans*, and contains approximately 2000 cells, subdivided in five types. The centre of the islet is composed mainly of β-cells that produce and secrete insulin; at the periphery, α, δ, and PP cells release glucagon, somatostatin and pancreatic polypeptide, respectively. Recently, 1% of islets cells have been unveiled to secrete the ghrelin hormone, thus constituting fifth type of cells (1).

Insulin plays a unique role in the maintenance of energy homeostasis and is therefore tightly controlled in order to maintain blood glucose in a physiological range. Short-term regulation of

insulin occurs mainly at the level of the secretory process, whereas maintenance of proper insulin stocks relies on the transcriptional and translational control of insulin synthesis.

A.1.2 Control of synthesis

The insulin gene encodes the precursor of the insulin polypeptide (proinsulin) chain and is composed of a single or two distinct genes, according to the species. Glucose increases preproinsulin mRNA through positive effects on its transcription rate and inhibition of its degradation. Insulin gene expression is mainly regulated by a 340 base pair region upstream of the transcription start site. This region contains cis-acting sequence motifs, including A3, E1 and C1 sites to which the transcription factors Pdx-1 (pancreatic and duodenal homeobox-1), NeuroD1 (neurogenic differentiation 1) and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homologue A) bind, respectively. These three factors act coordinately to stimulate insulin gene expression in response to elevated blood glucose (2). In addition, the velocity of preproinsulin translation is controlled by glycemia at multiple levels. Once expressed in the endoplasmic reticulum, the precursor undergoes post-translational proteolysis of the NH_2-terminal signal peptide. Proinsulin is converted into insulin by the proconvertases PC2 and PC3 in its journey along the secretory apparatus. The mature hormone is then packed into secretory granules or large dense-core vesicles dispersed throughout the cytosol or docked to the plasma membrane.

A.1.3 Control of secretion

The most important physiological regulator of insulin secretion is glucose. A mechanistic whereby glucose elicits insulin secretion has been referred as the triggering pathway (Fig. 1). Glucose enters the β-cell via the high K_m glucose transporter GLUT2 and is rapidly trapped into

glucose-6-phosphate by the β-cell-specific hexokinase, namely glucokinase. Phosphorylated glucose can then enter the glycolytic pathway, before going through the Krebs cycle and the electron transport chain. Glucose metabolism leads to the production of energy and increases the cellular ATP/ADP ratio. The unbalanced ATP/ADP ratio provokes the closure of the K^{+}_{ATP} channels and the subsequent depolarization of the plasma membrane. The loss of membrane potential triggers the opening of the voltage-dependent Ca^{++} channels (VDCC). The influx of calcium sets into motion the events leading to the exocytosis of secretory granules and the release of insulin into the extracellular milieu (Fig 1).

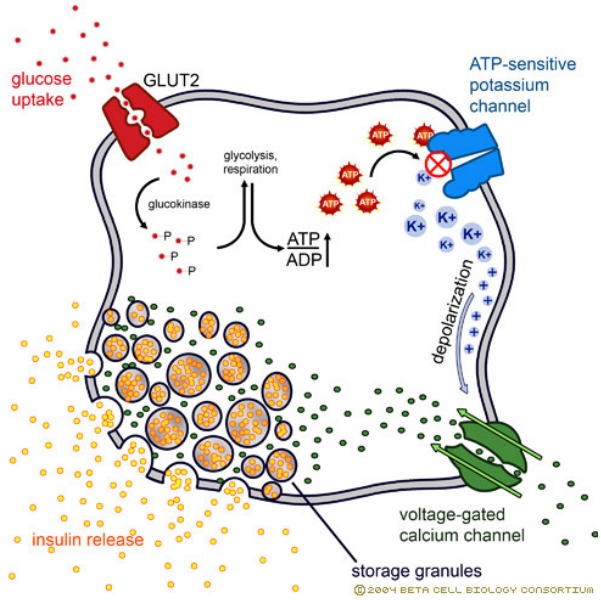

Fig1: Insulin secretion
Insulin secretion is triggered by a rise in glucose concentration. Glucose enters the cell via the glucose transporter GLUT2 and is phoshorylated by the hexokinase glucokinase, before being metabolized through glycolysis, the Krebs cycle and the respiratory chain. This results in an increased ATP/ADP ratio, leading to the closure of the K^{+}_{ATP} channels and membrane depolarization. The opening of the VDCC is favored by the change in membrane potential, ensuing in a net influx of calcium into the β-cell which triggers secretory granules fusion and insulin release (Adapted from the Beta-Cell Biology Consortium).

Under conditions of prolonged physiological glucose stimulation, the insulin secretion curve is biphasic. The release of insulin augments rapidly to a peak at 2-4 minutes, decreases between 10 and 15 minutes, and then progressively increases to reach a pseudo-plateau at 2-3 hours. The initial peak corresponds to the release of granules already docked to the membrane, referred as the first phase of insulin secretion or the “readily releasable pool”. The following increase of

insulin secretion is considered to represent the second phase and reflects the slow recruitment of a reserve pool of secretory granules and their transportation towards the plasma membrane. Although the triggering pathway is required to promote the two phases of secretion, a K^+_{ATP} independent pathway, also referred as an "amplifying pathway" has been highlighted to be further needed for the second phase. The exact mechanism of the pathway is still subject to debate, while several metabolites including, glutamate, NADPH, long chain acyl-CoA, Malonyl-CoA and GTP resulting from glucose and mitochondria metabolism, may serve as enhancing factors coupling glucose to insulin secretion (3).

A.2 Insulin action

The effects of insulin on its target organs are aimed at increasing glucose transport and restoring basal levels of circulating glucose and rely on the triggering of a complex signaling cascade (4).

The insulin receptor belongs to a family of tyrosine kinases and is composed of two α- and β-subunits. Binding of insulin by the α-subunits induces the tyrosine kinase activity of the β-subunits in the intracellular compartment and favors its autophosphorylation. Activated insulin receptor attracts and phosphorylates the family of insulin-receptor substrate (IRS 1-4) on tyrosine residues.

The enzyme phosphatidylinositol-3-kinase (PI(3)K) plays a central role in the metabolic actions of insulin. PI(3)K possesses SH2 domains in its p85 regulatory subunit which interact with phosphorylated tyrosine residues of the IRS proteins. The p110 catalytic subunit of the PI(3)K catalyses the phosphorylation of phosphatidylinositol-(4,5)-diphosphates ($PI(4,5)P_2$) into $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ which interact with the pleckstrin homology (PH) domain of signaling

proteins, including IRS proteins and Akt/PKB, and activates the serine/threonine kinase phosphoinositide-dependent kinase 1 (PDK1). In turn, PDK1 induces Akt/PKB by phosphorylation, thereby leading to the regulation of a variety of enzymes involved in the metabolism of glucose and synthesis of glycogen, proteins and lipids (Fig 2).

Two additional pathways are activated upon binding of insulin. First, the adapter protein Grb2 binds to phosphotyrosine residues of the IRS proteins. This interaction leads to the stepwise activation of Ras, Raf, MEK and the extracellular signal-regulated kinase (ERK), which induces a transcriptional programme enhancing cell growth and differentiation. Second, the tyrosine kinase domain of the insulin receptor activates the Cbl/CAP protein complex which interacts with proteins present in lipid rafts and potentiates mechanisms responsible for the uptake of glucose (Fig 2).

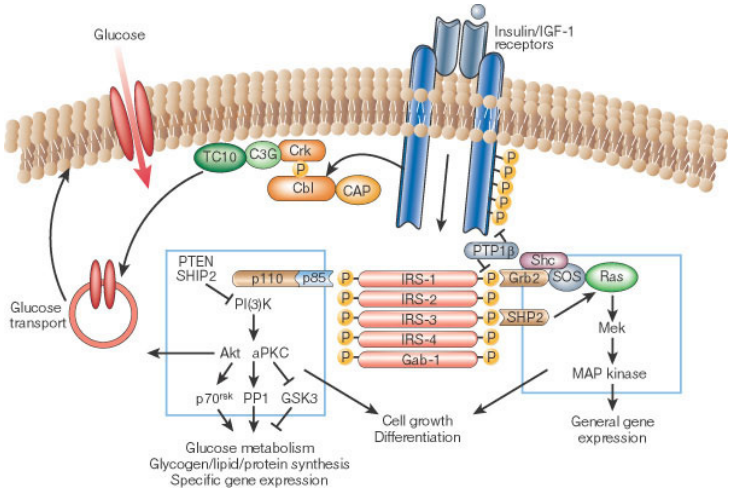

Fig 2: Insulin signaling
The insulin receptor possesses a tyrosine kinase domain which autophosphorylates upon binding of insulin. Activated insulin receptor catalyses the phosphorylation of signal proteins such as the IRS family and Cbl. In turn, these proteins recruit and activate diverse cascades, such as PI(3)K/Akt, Cbl/CAP complex and Grb2/Ras/Raf/MEK/ERK. These pathways coordinate the regulation of the metabolic and mitogenic effects of insulin (Adapted from (4)).

A.2.1 Liver

In hepatocytes, insulin favors glucose utilization and storage as fatty acids and glycogen, while inhibiting hepatic glucose production. The hormone induces the production of glycogen by activating the enzyme glycogen synthase (GS). This relies on the dephosphorylation of GS through inhibition of the glycogen synthase kinase-3 (GSK-3) by phosphorylated Akt and recruitment of the protein phosphatase 1 (PP1). In addition, insulin promotes the expression of key enzymes of the glycolytic pathway, such as glucokinase (GK), phospho-fructokinase-1 (PFK1) and pyruvate kinase (PK), thereby enhancing glucose degradation through glycolysis. Insulin also stimulates the synthesis of free fatty acids (FFA) via expression of the fatty acid synthase (FAS) and the acetyl-coA-carboxylase (ACC). This induction in glycolytic and lipogenic gene expression is regulated by an increased activity of the steroid regulatory element-binding protein (SREBP-1c).

On the other hand, insulin decreases the production of glucose by the liver. Indeed, the hormone diminishes the activity of enzymes implicated in gluconeogenesis by reducing the levels of the second messenger 3'-5'-cyclic adenosine monophosphate (cAMP). Additionally, the expression of gluconeogenetic enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase is decreased in states of hyperinsulinemia. This inhibition is mediated by a series of transcription factors including hepatic nuclear factor-4 (HNF-4), the forkhead protein family (Fox) and PPARγ co-activator 1 (PGC-1). Finally, insulin indirectly slows down hepatic glucose production by suppressing the systemic availability of gluconeogenic substrates, such as free fatty acids (4).

A.2.2 Muscle

Skeletal muscle is the main tissue responsible for insulin-stimulated glucose disposal (5). In myocytes, insulin promotes glucose captation, utilization and storage as glycogen.

The stimulation of glucose transport by insulin relies on the translocation of glucose transporter proteins from intracellular sites to the plasma membrane (6). The glucose transporter family is composed of different isoforms which exhibit a specific tissue distribution. The GLUT4 isoform accounts for the majority of insulin-stimulated glucose transport in muscle and adipose tissue (7). In basal conditions, GLUT4 is located on vesicles recycling between intracellular stores and the plasma membrane. Upon insulin stimulation, the mobility of GLUT4 vesicles along microfilament tracks is increased, plasma membrane architecture is modified and fusion of GLUT4 vesicles with the membrane is favored. This supports the exocytosis of GLUT4 vesicles and increases the presence of the transporter protein in the plasma membrane and the subsequent uptake of glucose (4). The translocation of GLUT4 is controlled by different signaling proteins. The protein kinase Akt, the atypical protein kinase C (PKC) λ and ζ and the Cbl/CAP protein complex are involved in the stimulation of GLUT4 exocytosis by insulin (4;8-10).

Similarly to its effects in hepatocytes, insulin stimulates glucose utilization and storage in myocytes via induction of glycogen synthase activity and glycolytic enzymes expression.

A.2.3 Adipose tissue

An increase in the circulating levels of insulin induces glucose uptake by adipocytes and lipid synthesis, and inhibits lipolysis. The mechanisms responsible for the augmentation of glucose captation by adipose tissue upon insulin stimulation are similar to those in skeletal muscle and rely on the translocation of GLUT4 vesicles to the plasma membrane (4;11).

The major inducers of lipolysis in adipocytes are the catecholamines which increase intracellular levels of cAMP upon binding to their membrane receptors. Up-regulation of the second messenger cAMP induces the protein kinase A (PKA) which in turn activates the hormone-sensitive lipase (HSL). Insulin potently inhibits lipolysis in adipocytes by decreasing cAMP levels, through activation of the enzyme phosphodiesterase-3 (PDE-3) (4;11). Additionally, this repression of FFA release has an indirect inhibitory effect on the production of glucose by the liver. Indeed, the reduced concentration of FFA in the plasma diminishes the delivery of gluconeogenic substrates to the liver and represses therefore the hepatic glucose production.

Similarly to its effect in the liver, insulin supports the synthesis of fatty acids in adipose tissue via increased activity and expression of the steroid regulatory element-binding protein-1c (SREBP-1c). This transcription factor controls the expression of lipid synthetic enzymes, including acetyl-CoA-carboxylase (ACC) and fatty acid synthase (FAS). Furthermore, incorporation of fatty acids into triglycerides is ameliorated by insulin via activation of the enzyme glycerol-3-phosphate-acyltransferase (12).

B Type 2 diabetes

B.1 Definition and etiology

Diabetes mellitus represents a group of metabolic diseases of heterogeneous etiology characterized by one common manifestation: hyperglycemia. In June 1997, an international expert committee has released a report with new recommendations for the classification and diagnosis of diabetes mellitus (13). Thus, in clinical practice, diabetes diagnosis is based primarily on fasting plasma glucose (FPG) concentration $\geq$ 7 mmol/l on two separate occasions.

Other options for diagnosis include a 2 h post-glucose load (75g glucose) or random postprandial plasma glucose level of ≥ 11.1 mmol/l. After a long duration of the metabolic imbalance, diabetic complications, such as neuropathy, retinopathy, nephropathy and atherosclerosis, may additionally develop. In the turn of this century 171 million individuals were estimated to have diabetes worldwide, and this is expected to increase over 350 millions by 2030. The prevalence of diabetes and its related complications constitute therefore a social and economic burden for our modern society. The etiological classification of diabetes includes mainly type 1, type 2, "other specific types" and gestational diabetes. Amongst them, type 2 diabetes (T2D) is the most common form, representing 90% of all diabetes cases.

The alteration in the glycemic control in case of type 2 diabetes ensues from two pathogenic processes: insulin resistance and β-cell dysfunction. In the context of sustained fuel supply and associated obesity, insulin resistance develops when target organs of insulin, such as liver, muscle and adipose tissue become less sensitive to the action of this hormone. Normal pancreatic β-cells respond to this impairment in insulin sensitivity by a compensatory hypersecretion of hormone, in order to maintain normal glucose tolerance. At some point however, pancreatic β-cells malfunction and fail to compensate for insulin resistance, leading to persistent hyperglycemia and overt type 2 diabetes (14).

B.2 Impairment in insulin action: insulin resistance

The term insulin resistance applies to the decline in the effects of insulin on glucose uptake and utilization in target organs. Physiological variations in insulin sensitivity occur during the normal life cycle, with insulin resistance being observed during puberty (15), pregnancy (16) and ageing (17). On the other hand, insulin sensitivity can be improved by lifestyle modifications, such as

enhanced physical activity (18) and carbohydrate intake (19). Importantly, insulin resistance is the initial measurable defect in patients who are destined to develop T2D. This alteration is present in the majority of patients with glucose intolerance or overt T2D and is strongly associated with obesity (20;21).

Loss of insulin responsiveness dramatically affects glucose homeostasis by impairing glucose disposal by muscle and adipose tissue and insulin suppression of hepatic glucose production. However, normoglycemia is, in the beginning maintained by a compensatory hypersecretion of insulin by the pancreatic β-cells. Sustained hyperglycemia and overt T2D only develop when these cells suffer from dysfunction and fail to meet the metabolic demand of insulin (14).

B.2.1 Liver

Because of its important contribution to glucose homeostasis, development of insulin resistance in the liver strongly impacts on glycemic control and progression of T2D.

The main consequence of hepatic insulin resistance is the impaired suppression of glucose production by this hormone (22). Indeed, insulin-insensitive hepatocytes exhibit increased gluconeogenesis and glycogen degradation. Since the liver is the major source of glucose after an overnight fast, loss of hepatic insulin sensitivity strongly participates to the development of fasting hyperglycemia in T2D. Various alterations in the cascade of insulin signaling could account for the loss of hepatic insulin responsiveness (23). Reduced expression and inhibitory serine phosphorylation of signaling proteins, such as the family of IRS proteins and induction of phosphatases enzymes may impair transmission of the insulin signal.

In addition to the elevation in hepatic glucose production, the insulin-resistant liver is characterized by an excessive accumulation of triglycerides – a process referred as hepatic steatosis. The mechanisms responsible for the exaggerated deposit of fat are linked to an increased delivery of FFA from insulin-insensitive adipose tissue and de novo lipid synthesis in the liver itself (24). This disruption could ultimately lead to the emergence of nonalcoholic fatty liver disease (NAFLD), which represents the most common cause of liver abnormality in westernized societies.

B.2.2 Muscle

Since skeletal muscle is the principal tissue responsible for insulin-induced glucose uptake (5), the loss of insulin responsiveness in this organ strongly contributes to impaired glucose homeostasis in T2D. Indeed, insulin-resistant myocytes exhibit a reduced transport of glucose upon insulin stimulation (25) and an alteration in glycogen synthesis (26).

The reduction in glucose uptake in insulin-insensitive skeletal muscle ensues from alterations in the insulin signaling cascade and impairment in GLUT4-mediated glucose transport. The deterioration in insulin signaling relies on the reduced phosphorylation of insulin receptor, IRS proteins and perturbed activity of PI(3)K, as observed in the skeletal muscle of T2D (27). The defect in GLUT4-mediated glucose uptake is the consequence of reduced presence of the glucose transporter at the plasma membrane. This decline in GLUT4 at the membrane issues from an impaired translocation of GLUT4 vesicles but not from a reduced expression of the transporter protein (28).

Similarly to the liver in T2D, insulin-resistant skeletal muscle presents an elevation in triglycerides accumulation (29). This augmentation in intramuscular lipid is associated with a deterioration in the capacity of oxidative and glycolytic enzymes (27).

B.2.3 Adipose tissue

The adipose tissue of T2D is characterized by a diminution in glucose uptake and an absence of suppression of FFA release by insulin. Similarly to its transport in myocytes, glucose entry in the insulin-resistant adipocytes is decreased as a consequence of a perturbation in insulin signaling (30). Adipocytes in T2D have a marked reduction in the insulin-stimulated IRS proteins phosphorylation and PI(3)K activity. However, on the contrary to muscle, adipose tissue demonstrates a decline in the expression of the glucose transporter GLUT4 which contributes to the loss of glucose uptake by adipocyte (30).

Furthermore, insulin-insensitive fat cells are less susceptible to the inhibition of HSL activity by insulin. This mechanism leads to an enhanced release of FFA in plasma from triglyceride stocks, which in turn contributes to the worsening of glycemic control in T2D. The excess of circulating FFA stimulates gluconeogenesis and glucose release by the liver, thereby further increasing blood glucose levels. In parallel, the plasma FFA accumulate outside the adipose depots, including skeletal muscle and liver, where they aggravate insulin resistance (14).

Beside their role as storage depot for lipids, fat cells produce and secrete number of hormones, collectively referred as adipokines. For instance, leptin, adiponectin, resistin and others are released by the adipose tissue and could profoundly influence metabolism and energy expenditure (4). Importantly, in insulin resistant conditions and associated obesity, the pattern of

secretion of these hormones is perturbed; an alteration which is thought to promote the impairment in insulin sensitivity and glycemic control.

B.2.4 Genetic factors

T2D is a polygenic disorder in which multiple genes contribute to a general predisposition to develop the disease. Genome-wide analyses have so far identified 23 loci associated with T2D (31;32). The majority of these susceptibility loci can be directly mapped to protein of the pancreatic β-cell. Interestingly, only few of them were associated with increased risk for insulin resistance. For instance, PPARγ, IRS1, Glucokinase Regulator (GCKR) and Insulin-like Growth factor 1 (IGF1) are related with insulin action, and functional studies have demonstrated that some of them influence insulin sensitivity (33).

B.2.5 Environmental factors

Whereas limited numbers of genes have been associated with increased susceptibility to insulin resistance, numerous metabolic alterations have been correlated with the obesity-related loss of insulin sensitivity.

B.2.5.1 Hyperinsulinemia

As previously mentioned, pancreatic β-cells counterbalance the loss of insulin responsiveness in target tissues by a hypersecretion of hormone, thereby maintaining normoglycemia. However, this condition of compensation is not without consequence and the subsequent hyperinsulinemia exacerbates the insulin resistant state. Elevated insulinemia further decreases insulin sensitivity by down-regulating insulin receptor protein, IRS proteins and other insulin signaling molecules

(23). In addition, hyperinsulinemia worsens hepatic steatosis via stimulation of de novo lipogenesis in the liver through activation of the transcription factor SREBP-1c (23).

B.2.5.2 Fatty acids

As a consequence to the loss of insulin-mediated suppression of HSL activity, insulin-resistant adipose tissue releases abnormally elevated levels of FFA into the plasma. These circulating fatty acids accumulate outside the fat depots such as in the liver and skeletal muscle, where they perturbate the action of insulin.

In the liver, the excess of FFA is redirected into cytosolic lipid species such as triglycerides (TG), diacylglycerol (DAG) and cermides, as a result of the reduction in fatty acids β-oxidation. This alteration is thought to be regulated by the glucose-induced increase in malonyl-CoA, which inhibits the import of fatty acids into the mitochondria via the carnitine palmitoyltransferase-1 (CPT-1) protein. Such accumulation of lipid species sets the stage for the development of hepatic steatosis and associated loss of insulin sensitivity (34). In contrast in skeletal muscle, the surplus lipids not only accumulate in cytosol as TG, DAG and ceramides but are transferred to mitochondria for β-oxidation. However, in absence of exercise, this augmentation in fatty acid oxidation is not matched by an elevation in Krebs cycle activity. As a consequence, lipid derivatives concentrate in the mitochondria engendering mitochondrial stress and contributing to impaired insulin responsiveness (14).

B.2.5.3 Inflammatory cytokines

The adipose tissue of obese subjects is characterized by a low-grade inflammation and recruitment of macrophages (35). Whereas the adipose production of inflammatory cytokines is

under debate, the recruited macrophages release TNF-α and IL-6, thereby establishing a state of systemic low-grade inflammation. The enhanced circulating levels of inflammatory cytokines contribute to the loss of insulin sensitivity in target tissues via activation of the transcription factor Nuclear Factor-κB (NF-κB) (14).

B.2.5.4 Adipokines

In addition to their capacity to store lipids, adipocytes have the ability to produce and release numerous bioactive peptides, known as adipokines. These molecules encompass the retinol-binding protein-4 (RBP4), resistin, leptin, adiponectin and others. Obesity-related expansion of adipose tissue is associated with an alteration of the production of adipokines, which can profoundly affect the ability of organs to respond to insulin (14). For instance, the release of the adipose tissue-specific collagen-like protein adiponectin is reduced in obesity – a decrease which correlates with the degree of insulin resistance (36). Adiponectin is categorized as an "anti-diabetogenic" molecule, based on its capacity to reduce triglyceride synthesis, stimulate β-oxidation and enhance insulin action in both skeletal muscle and liver (14). It is therefore not surprising that mice knock-out for adiponectin develop severe insulin resistance upon administration of a high fat diet (37). The insulin-sensitizing property of this adipokine relies on its ability to activate 5'-AMP-activated protein kinase (AMPK), an enzyme that responds to a decrease in ATP/AMP ratio by activating both glucose and fatty acid oxidation (14).

B.3 Impairment in insulin secretion: β-cell dysfunction

Healthy β-cells are able to counter loss of insulin sensitivity by a compensatory hypersecretion of the hormone, as a result of increased β-cell mass and insulin release capacity. This process is so efficient that normal glucose tolerance can be maintained for a long time. At some point,

however, β-cells fail to compensate for insulin resistance and glucose intolerance and to the extreme T2D develop. Consequently, β-cell dysfunction represents a key step in the progression of the disease, allowing for transition between an asymptomatic state to overt diabetes (14).

Evidence for an early alteration of β-cell secretory capacity in patients with glucose intolerance and T2D has accumulated over years. Indeed, both phases of insulin release have been demonstrated to be reduced in glucose intolerant individuals in response to intravenous glucose infusion. Whereas the non-glucose secretagogue arginine is able to elicit insulin release in diabetic patients, the magnitude of secretion is however lower than in healthy individuals (38). In addition, pancreatic islets from cadaveric donors with type 2 diabetes are unable to augment insulin secretion during an *in vitro* hyperglycemic stimulation (39). This defect of insulin secretion is associated with damaged secretory machinery and impaired glucose metabolism. Such observations are reproduced in some animal models that mimic human T2D such as the Goto-Kakizaki (GK) rats (40).

In addition to the functional defects of the β-cells, reduction in their mass has been suggested to contribute to the progression of β-cell dysfunction. Control of the β-cell mass relies on the balance between β-cell replication and apoptosis, as well as differentiation of new islets from pancreatic ducts. Perturbation of the pathways of β-cell generation or increased β-cell death could reduce β-cell mass. Because of the difficulties to obtain pancreatic tissue from humans, there are only few studies of β-cell mass in diabetic patients. Morphometrical analyses of islets from T2D reported a 63% loss of β-cell volume compared with weight-matched controls. Moreover, this reduced β-cell volume is not the consequence of a decreased cell proliferation, but is associated with an increased β-cell apoptosis (41). However, although the number of β-cells is clearly

reduced, this degree of β-cell loss is not sufficient to fully account for the change in secretory function. *In vitro* and *in vivo* functional assessments of living islets show a marked decrease in secretion of insulin compared to islets from non diabetic individuals.

B.3.1 Genetic factors

While several rare monogenic forms of diabetes, including maturity-onset diabetes of the young (MODY), have been described, the genetic basis of T2D appears to be much more complex. T2D is viewed as a disease of polygenic origin in which multiple different genes contribute to a general predisposition to develop the disease. However, emergence of high-throughput genotyping sequencing methods and systemic screening of cohorts of thousands of patients have allowed the identification of several candidate genes for β-cell failure in T2D. Compelling all the data from the literature, 23 loci are associated with T2D so far. Amongst them, *Peroxisome proliferator-activated receptor gamma* (*PPARγ*), *KCNJ11* (*Kir6.2*), *transcription factor 7–like 2* (*TCF7L2*), *insulin-like growth factor 2 mRNA binding protein 2(IGF2BP2), CDK5 regulatory subunit associated protein 1-like 1 (CDKAL1), hematopoietically expressed homeobox (HHEX) and Zinc transporter 8 (SLC30A8)* are expressed in β-cells, and at present, functional studies have shown some of them playing a key role in β-cell function (31;32).

B.3.2 Environmental factors

The diabetic genotype is typically responsible for a part of the vulnerability of islets and for a predisposition for insulin resistance and T2D. However, pancreatic β-cell dysfunction and overt diabetes only develop once susceptible individuals have been recurrently exposed to environmental stressors.

B.3.2.1 Gluco- and glucolipo- toxicity

The term “gluco-toxicity” refers to the adverse effects of chronic exposure of pancreatic β-cells to elevated blood glucose. The piece of evidence that supports the detrimental effects of chronic elevation of glucose on insulin secretion comes from an investigation that has been realized in humans. Consecutive administrations of glucose by intraveinous injection in healthy individuals elicit a loss of glucose-induced insulin secretion (42). This observation suggests that chronic hyperglycemia, which could result from a combination of glucose intolerance and postprandial glucose excursions, would promote and/or worsen T2D development.

The molecular mechanisms through which chronic elevation of glucose exerts adverse effects on β-cells have been deciphered. Chronic hyperglycemia evokes impairment in both insulin synthesis and secretion. Alteration of insulin expression is caused by a reduced activity of the two major β-cell transcription factors, pancreatic-duodenum homeobox-1 (PDX-1) and rat insulin promoter element 3b1 (RIPE 3b1/MafA) (43). Reduction in the activity of these factors and loss of the β-cells secretory capacity is depicted to the generation of reactive oxygen species (ROS) (Fig. 3, (44)). Treatment with the antioxidant N-acetylcysteine (NAC) protects β-cells against the harmful effects of high glucose. This process which is referred to as oxidative stress is, at least partially, responsible for the modification in the transcriptional factors (43). Finally, it has been repeatedly reported that the prolonged exposure of rodent and human islets to elevated glucose induces β-cell apoptosis and could contribute to the loss of β-cell mass observed in islets from T2D patients (45).

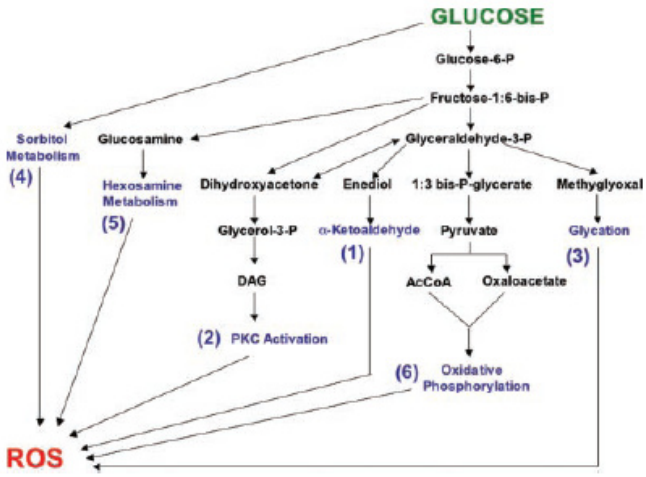

Fig 3: Six biochemical pathways along which glucose metabolism can form ROS
Under physiologic conditions, glucose primarily undergoes glycolysis and oxidative phosphorylation. Under pathologic conditions of hyperglycemia, excessive glucose levels can swamp the glycolytic process and inhibit glyceraldehyde catabolism, which cause glucose, fructose-1,6-bisphosphate, and glyceraldehyde-3-P to be shunted to other pathways: *1*, enolization and ketoaldehyde formation; *2*, PKC activation; *3*, dicarbonyl formation and glycation; *4*, sorbitol metabolism; *5*, hexosamine metabolism; and *6*, oxidative phosphorylation. *DAG*, diacylglycerol (Adapted from (44)).

By analogy to the paradoxically adverse effects of chronic hyperglycemia, free fatty acids (FFA), which are vital nutrients for β-cells, hamper their physiology when chronically administered. A four days infusion of intra-lipids in individuals with a family history of diabetes elicits loss of glucose-induced insulin secretion (46). *In vitro*, the sustained exposure of pancreatic β-cells to FFA reduces glucose-induced insulin secretion and affects insulin gene expression, together with a reduction in PDX-1 activity (43). Finally, excess in FFA provokes β-cell death by apoptosis (47) and generates oxidative stress (48).

Interestingly, lipid-induced alterations in β-cell function can be potentiated by supraphysiological concentrations of glucose. In contrast, at low glucose levels, the excess of FFA is efficiently metabolized and does not harm the β-cell. The synergistic impairment of β-cell function by glucose and FFA is referred to as the "glucolipotoxicity" and has been verified in both *in vitro* and *in vivo* systems. The simultaneous presence of elevated levels of glucose and FFA catalyzes the accumulation of malonyl-CoA, a potent negative regulator of the carnitine-palmitoyl-transferase-1 (CPT-1). Inhibition of FFA transport into the mitochondria via CPT-1 results in the

cytosolic accumulation of long-chain fatty acyl-CoAs (LC-CoA), which are proposed as mediators of the adverse effects of persistent elevations of FFA (43).

B.3.2.2 Pro-inflammatory cytokines

Recent studies on the role of inflammation in chronic non-immune diseases, such as obesity and atherosclerosis, have shed light onto the close association between inflammatory markers and the metabolic alterations in T2D and the contribution of inflammation agents to β-cell dysfunction and insulin resistance. The sources of pro-inflammatory cytokines that negatively affect β-cells are multiple and encompass adipocytes, macrophages, endothelial cells, as well as pancreatic islet cells. The three most deleterious cytokines favoring β-cell death are interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) and interferon γ (IFNγ). TNFα is secreted by macrophages and adipose tissue and its receptor-mediated recognition triggers the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK, also known as MAPK8) and p38 (MAPK11-14), as well as the Nuclear Factor κB (NFκB) pathway. IFNγ is released by T-helper cells and induces the Janus tyrosine kinase (JAK)/Signal Transducer and Activator of Translation (STAT) path. Finally, IL-1β activates the same effectors pathways as TNFα and is liberated by activated macrophages and, paradoxically, under some circumstances by β-cells (45).

B.3.2.3 Modified LDL and HDL: additional environmental stressors for β-cells

Individuals with T2D have an increased risk to develop cardiovascular diseases. This association has led to the hypothesis that diabetes and cardiovascular affections share common precursors. Low levels of circulating high-density lipoproteins (HDL), associated with augmented concentrations of modified low-density lipoprotein particles (LDL), particularly oxidized LDL,

are associated with a higher risk for T2D patients to develop atherosclerotic cardiovascular disease. Numerous clinical studies reflect this correlation. In the prediabetic stage, during which hyperglycemic excursions increase abnormally, the high blood glucose appears as an independent risk factor for cardiovascular diseases (49). According to the United Kingdom Prospective Diabetes Study, LDL-cholesterol is the most important determinant for development of cardiovascular disease in T2D (50). *In vitro* and *in vivo* experiments show that hyperglycemia can potently modify LDL, particularly by generating glycated LDL, a particle that is more susceptible to oxidative modifications. A clear increase in the ratio of oxidized LDL over native is systematically observed in plasma of patients with T2D, whereas the total plasma levels of LDL-cholesterol are often modestly increased (51). HDL and paraoxonase (PON1) play an anti-oxidant role, insuring a certain detoxification rate of oxidized LDL. The plasma concentration of HDL and PON1 is reduced in diabetes, thus providing an additional mechanism for oxidization of LDL (52). A role for oxidized LDL as a risk factor for developing diabetes is further supported by the fact that a long term follow-up study, which has prospectively investigated the risk factors of people at work (employees of companies) for developing diabetes over 4-10 years, does not confirm an association of diabetes with an increased concentration of native LDL (53). Indeed, progression of diabetes correlates with a decrease in HDL plasma level while the concentration of LDL does not increase with hyperglycemia, suggesting rather that oxidization of LDL plays a role in the pathogenesis of β-cell failure (53). The presence of oxidized LDL in plasma of patients with T2D is further provided by the identification of auto-antibodies directed against oxidized LDL in these patients (54).

Although oxidized LDL levels are clearly increased and HDL low in the plasma of glucose intolerant and T2D patients, the firm demonstration of their contribution to the progression of β-

cell failure and subsequent acceleration of overt diabetes remains to be made. However, several lines of evidence have been accumulated in favor of the participation of lipoproteins in β-cell failure. First, once oxidized in the subendothelial space of the vessel wall, a portion of lipoproteins are released in the blood flow. These particles possess a size able to diffuse into the pancreatic islets via fenestrated capillaries. Secondly, effective expression of receptors dedicated to cellular uptake of LDL particles, such as LDL receptor (LDLR) and LDLR-related protein (LRP) is observed in mouse islets and β-cells, as well as the HDL-binding Scavenger Receptor BI (SR-BI) and Apolipoprotein E Receptor 2 (ApoER2) (55). The scavenger receptor CD36 for oxidatively modified LDL is present on the plasma membrane of insulin-secreting and rat islets cells (56). In line with the expression of their affiliated receptors, LDL and HDL are effectively recognized and endocyted by human and rodent islets, as well as by mouse insulin-secreting cells (55;57). In addition, binding and internalization of oxidized lipoproteins by β-cells has been reported (56). Chronic exposure of the insulin-producing HIT-T15 β-cell line to physiological cholesterol concentration of oxidized LDL (< 2.6 mM), but not native particles reduces insulin biosynthesis and secretion (56). Adverse effects of unmodified lipoproteins only become apparent at supraphysiological dosages or after serum starvation-induced LDLR up-regulation (55;58).

B.4 Role of the transcription factors modulated by cAMP in β-cell failure and insulin resistance

To adapt to environmental cues such as hormonal or nutrients changes, most of cells are equipped with receptors and signaling cascades that rapidly transduce signals, thereby triggering changes in gene expression and cellular functions. To achieve efficient coupling of extracellular stimulus to cellular outcomes, several second messengers are particularly crucial. Amongst them is cyclic

adenosine monophosphate (cAMP) that plays a role in a considerable number of physiological processes, including energy metabolism, cellular proliferation and neuronal signaling (59;60).

Adenylate cyclase is the main enzyme responsible for the regulation of intracellular levels of cAMP. This enzyme is modulated by various extracellular signals and their affiliated receptors and G-proteins. cAMP interacts with the two regulatory subunits of PKA, releasing the catalytic parts of the enzyme. The activated catalytic entities ultimately phosphorylate and stimulate a specific set of transcription factors responsible for the control of genes expression through cAMP-inducible promoter elements. These promoter responsive sequences are referred as cAMP Responsive Element (CRE) and the first factor identified to bind these elements was the CRE-Binding Protein (CREB). The PKA favors the transcriptional activity of CREB by phosphorylation on a serine residue at position 133. Two additional members of the CREB family were subsequently discovered: the Activating Transcription Factor-1 (ATF1) and the CRE Modulator (CREM). These three classes of transcription factors contain consensus PKA phosphorylation sites and interact with DNA as dimers via a basic leucine zipper motif. Finally, the termination of the cAMP signal involves an atypical member of the CREM family. The Inducible Cyclic AMP Early Repressor (ICER) is up-regulated by cAMP itself through an alternative promoter within the CREM gene which contains four CRE sites (61). ICER contains the basic leucine zipper domain of the CREM, but lacks the activating domain. Consequently, ICER can bind the CRE but it is unable to transactivate gene expression. As a passive repressor ICER will compete with the activating transcription factors of the CREB, CREM and ATF1 family and will therefore facilitate the restoration of basal expression levels of cAMP-responsive genes (59;60).

The interplay between ICER and the CREB, CREM and ATF1 transcriptional activators is critical for the regulation of the production and release of neurotransmitters and hormones including, β-endorphin, growth hormone (GH), prolactin (PRL) or adenocorticotropic hormone (ACTH) produced by anterior pituitary cells (62). This hormonal release plays a pivotal role in coordinating the physiologic adaptation of the hypothalamo-pituitary-adrenal axis to stress. In this case, stress is defined as physiological changes that accompany increased physical and psychological demands. Increasing evidence suggests that ICER plays a regulatory role in hormone release in pituitary cells. Consistently, targeted suppression of the CREM gene in mice, leading to the loss of ICER expression, results in a chronic increase in plasma β-endorphin (63). A model of pituitary corticotroph AtT20 cell line overexpressing ICER has been developed to measure the effects of this transcriptional repressor on hormone secretion (64). Ectopic expression of ICER blocks the synthesis of ACTH at post-translational levels and alters stimulus-induced secretion of this hormone (64). The role of CREB and ICER is critical for glucose homeostasis. In β-cells, the cAMP pathway contributes to the potentiation of glucose-stimulated insulin gene expression by incretin hormones (65). In liver, the CREB/ICER network promotes glucose homeostasis during fasting. This is achieved by regulating the expression of genes from the neoglucogenic program (66). In adipocytes, the cAMP signalling controls the production of the insulin sensitizer adiponectin (67).

B.4.1 Dysregulation of CREB in response to diabetic environmental stressors

The CREB activators play a critical role in tissues involved in pathogenesis of diabetes. Because they are rapidly activated by changes in extracellular environment and devoted to trigger cellular outcomes, dysregulation in their function was suggested to be involved in β-cell failure. The

dysfunction of pancreatic β-cells evoked by the chronic exposure to elevated concentrations of glucose is associated with a reduction in CREB activity, due to exacerbated proteosomal degradation of these transcription factors. Restoration of CREB levels protects β-cell from the adverse effects of chronic hyperglycemia (68). Accordingly, mice engineered to overexpress a dominant negative mutant of CREB specifically in the β-cell develop diabetes, as a consequence of a massive loss of β-cell mass by apoptosis (69). The molecular mechanism responsible for the alteration in CREB activity under diabetic conditions has been brought to light and relies on the transcriptional repressor ICER. Indeed, augmented expression of this transcription factor is observed in pancreatic β-cells chronically exposed to elevated concentrations of glucose or palmitate and in the islets of rat with T2D (70;71). Importantly, mice with a forced expression of ICER in β-cells suffer from severe diabetes (72). The dramatic loss of glucose homeostasis in these mice is associated with a failure of insulin biosynthesis and reduced β-cell mass (72). Finally, the contribution of ICER to the impairment in insulin secretion evoked by chronic high glucose levels or palmitate has been documented to involve the capacity of the transcriptional repressor to silence the expression of the exocytotic genes Rab3a, Rab27a and their effectors Slp4 and Noc2 (73), as well as the gap-junction protein connexin 36 (74). These observations emphasize therefore the role of the CREB pathway in the proper function pancreatic β-cells and the implication of the transcriptional repressor ICER in the progression of β-cell dysfunction in T2D.

B.4.2 Role of CREB in insulin resistance

The cAMP signaling is, in addition, involved in the progressive loss of insulin sensitivity. In fact, the invalidation of CREB in the liver protects from hepatic steatosis and insulin resistance in diabetic rodent models, such as the ZDF rats, the streptozotocin-treated and high fat diet (HFD)-

fed rats, as well as the ob/ob mice. (75). Furthermore, CREB activity in the adipose tissue has been reported to influence whole body insulin responsiveness. The CREB pathway is constitutively stimulated in fat cells of HFD-fed mice and db/db mice, where it contributes to insulin resistance by inducing the Activating Transcription Factor-3 (ATF3). In turn, this repressor suppresses the expression of the glucose transporter 4 (GLUT4) and the insulin-sensitizer hormone adiponectin (76). Transgenic overexpression of a dominant-negative CREB in adipocytes under obese conditions restores insulin sensitivity and blocks hepatic steatosis and adipose tissue inflammation (76).

II Objectives

II Objectives

A Unveiling and deciphering the role of oxidized LDL and HDL in β-cell failure

A role for oxidized LDL-cholesterol particles in the progression and pathogenesis of diabetes is more and more clear. Several lines of evidence indicate a potential effect of these particles in β-cells. These cells can internalize oxidized as well as native LDL and HDL particles through scavenger receptors. Additionally, experiments in an insulin-producing cell line show the deleterious effects of oxidized LDL on β-cell function. Consequently, the first aim of the present thesis is to better understand the molecular mechanisms of lipoproteins in β-cell dysfunction. Precisely, the contribution of the diabetogenic transcriptional repressor ICER and the generation of oxidative stress to the alteration of β-cell function evoked by modified lipoproteins will be assessed.

B Dissecting the molecular mechanism responsible for adipose tissue dysfunction elicited by CREB

The CREB pathway is involved in the deterioration of glucose homeostasis in T2D and particularly in the loss of insulin sensitivity. The activity of these factors is up-regulated in the adipocytes under obese conditions, where it favors the silencing of GLUT4 and adiponectin via activation of the transcription factor ATF3. As a consequence, the second aim of this thesis is to clarify the causes of the abnormal CREB activity in obesity and to elucidate the potential contribution of the transcriptional repressor ICER in this pathological process.

III Results

A A role for JNK pathway in β-cell dysfunction evoked by oxidized LDL

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A A role for JNK pathway in β-cell dysfunction evoked by oxidized LDL

A.1 Introduction

Frank hyperglycemia and T2D develop in patients whose pancreatic β-cells fail to secrete enough insulin to compensate for insulin resistance (14). β-cell dysfunction is characterized by a decline in the number of β-cells and their capacity to produce and release insulin. Environmental stressors, such as chronic elevation of glucose and FFA contribute to β-cell insult by persistently increasing intracellular levels of ROS – a process termed oxidative stress. The main mitogen-activated protein kinase associated with the generation of oxidative stress is the JNK kinases, which lead to the activation of the Activator Protein-1 (AP-1) transcriptional complex and subsequent loss of preproinsulin gene expression and β-cell survival (77). The scaffold protein Islet Brain 1 (IB1) is an important regulator of the JNK pathway which prevents the activation of c-Jun, a member of the AP-1 transcription complex. Interestingly, reduction in IB1 content contributes to the loss of insulin expression and β-cell survival under diabetic conditions (78;79).

Patients with T2D have a perturbed lipid profile, with elevated plasma levels of oxidized LDL (oxLDL) over native LDL. This augmentation is associated with reduced concentrations of HDL and is considered as a major risk factor for patients to develop atherosclerotic cardiovascular diseases. In addition to their pro-atherogenic properties, several lines of evidence support the implication of oxLDL in the progression of β-cell dysfunction and development of T2D. The prolonged exposure of insulin-secreting cells to modified lipoproteins causes a decline in insulin

production and secretion and triggers β-cell apoptosis (56;80). Noteworthy, in others tissues, oxLDL can induce oxidative stress and activate JNK pathway and AP-1 transcriptional activity – a mechanism that ultimately leads to cellular apoptosis (81).

In view of these data, we hypothesized that JNK signalling is implicated in the perturbation of insulin biosynthesis and β-cell survival caused by oxLDL.

A.2 Results

The role of lipoproteins in the progression of β-cell dysfunction was first confirmed by determinating the effects of oxLDL on insulin biosynthesis and cell survival. To do so, the insulin-secreting cell line MIN6 B1 and rat and human isolated islets were cultured in presence of 2mM cholesterol of copper-oxidized or native LDL for 72h. Modified lipoproteins induced a significant reduction in insulin content, which was associated with decreased preproinsulin mRNA level and insulin gene promoter activity (Fig 1 and 2a). In the meanwhile, native LDL at a similar cholesterol concentration did not affect the insulin production capacity of β-cell (Fig 1). In addition, the percentage of β-cell apoptosis – assessed as the number of cell displaying a picnotic nucleus – was markedly augmented by the modified lipoproteins, but not by native LDL (Fig 6b). This increase in cell death was correlated with the loss of expression of the anti-apoptotic factor Bcl2 (Fig 6d).

The alteration in β-cell function and survival by oxLDL was associated with an activation of the JNK pathway, as reported by increased phosphorylation of the JNK target c-Jun, AP-1 transcriptional activity and c-Jun and c-Fos expression (Fig 3 and 4). Interestingly, the long-term induction of this signalling path was correlated with the disappearance of the scaffold protein IB1

(Fig 6a). The contribution of the JNK cascade to the β-cell dysfunction evoked by oxidized LDL was confirmed by the restoration of insulin mRNA levels and cell survival by the JNK inhibitor JNKi during chronic oxLDL treatment (Fig 5a and 6b-c). Interestingly, the anti-atherogenic particles HDL prevented development of β-cell failure by modified LDL. Indeed, insulin gene expression, IB1 content were augmented, whereas apoptosis was reduced in MIN6 cells and human islets exposed to oxLDL and HDL (Fig 7a, 8 and 9e-f).

In conclusion, these data provide evidence that oxidized, but not native LDL exert deleterious effects on insulin levels and β-cell survival by reducing IB1 content and activating the JNK pathway. Inhibition of this signalling cascade by the inhibitor JNKi or HDL counteracts the adverse consequence of oxLDL exposure on β-cell function.

A.3 Contribution

In this paper, the student was involved in the experimental data collection, being specifically in charge of the AP-1 transcriptional activity assays, the quantification of the mRNA of c-Jun, c-Fos, Bcl2 and the preparation of the lipoproteins and experimental media. In addition, the student took a significant role in the lead of the revision experiments and corrections. He drove the statistical analysis of the majority of the data and was implicated in the design of the experiments, the preparation of the figures and the writing of the manuscript.

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ARTICLE

Human high-density lipoprotein particles prevent activation of the JNK pathway induced by human oxidised low-density lipoprotein particles in pancreatic beta cells

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Abstract
Aims/hypothesis We explored the potential adverse effects of pro-atherogenic oxidised LDL-cholesterol particles on beta cell function.
Materials and methods Isolated human and rat islets and different insulin-secreting cell lines were incubated with human oxidised LDL with or without HDL particles. The insulin level was monitored by ELISA, real-time PCR and a rat insulin promoter construct linked to luciferase gene reporter. Cell apoptosis was determined by scoring cells displaying pycnotic nuclei.
Results Prolonged incubation with human oxidised LDL particles led to a reduction in preproinsulin expression levels, whereas the insulin level was preserved in the presence of native LDL-cholesterol. The loss of insulin production occurred at the transcriptional levels and was associated with an increase in activator protein-1 transcriptional activity. The rise in activator protein-1 activity resulted from activation of c-Jun N-terminal kinases (JNK, now known as mitogen-activated protein kinase 8 [MAPK8]) due to a subsequent decrease in islet-brain 1 (IB1; now known as MAPK8 interacting protein 1) levels. Consistent with the pro-apoptotic role of the JNK pathway, oxidised LDL also induced a twofold increase in the rate of beta cell apoptosis. Treatment of the cells with JNK inhibitor peptides or HDL countered the effects mediated by oxidised LDL.
Conclusions/interpretation These data provide strong evidence that oxidised LDL particles exert deleterious effects in the progression of beta cell failure in diabetes and that these effects can be countered by HDL particles.

Keywords Apoptosis · Diabetes · HDL · Insulin · JNK pathway · MAPK · Oxidised LDL · Pancreatic beta cells

Abbreviations

AP1	activator protein-1
AP1Luc	luciferase reporter construct driven by multimerised AP1 consensus sequences
Bcl2	B-cell leukaemia/lymphoma 2
GST	glutathione *S*-transferase
IB1	islet brain 1
JNK	c-Jun N-terminal kinases
Luc	luciferase reporter construct
MAPK8	mitogen-activated protein kinase 8
Rip	rat insulin II promoter
RIPE	rat insulin promoter element
RipLuc	rip linked to the luciferase reporter

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Introduction

A decline in the number of insulin-producing beta cells and/or their intrinsic ability to produce and/or secrete insulin contributes to the pathophysiology of type 2 diabetes [1]. It is now accepted that this beta cell inadequacy results in part from oxidative stress due to adverse effects of chronic elevation of glucose and free or non-esterified fatty acids [2–4]. Among the major mechanisms that have been associated with oxidative stress, is induction of the c-Jun N-terminal kinases (JNK, now known as mitogen-activated protein kinase 8 [MAPK8]) signalling pathway, leading to activation of activator protein-1 (AP1) transcriptional factors complex [5, 6]. Suppression of this pathway prevents the loss of preproinsulin gene expression and apoptosis [7, 8].

Islet-brain 1/JNK-interacting protein 1 (IB1; now known as MAPK8 interacting protein 1) is a mammalian scaffold protein involved in the regulation of the JNK pathway [9–11]. One of the outcomes of this regulation is to prevent the activation of c-Jun (now known as JUN or Jun oncogene), a transcription factor included in the AP1 transcriptional complex that directly represses production of insulin and induces beta cell apoptosis [11–14]. Reduction in IB1 content diminishes preproinsulin mRNA levels and renders the cells more sensitive to stress-induced programmed death [11, 15, 16]. The importance of IB1 levels in beta cells has been confirmed in human diabetes. A missense mutation (S59N) in the gene encoding IB1 has been found to co-segregate with diabetes in a French family with a monogenic form of type 2 diabetes. Ex vivo, this mutation reduces the stability of IB1, leading to decreased insulin promoter activity and acceleration of the rate of cell apoptosis [11, 17].

Elevated levels of oxidised LDL-cholesterol, together with low HDL-cholesterol, are typical symptoms of diabetic dyslipidaemia and risk factors for prediabetic and diabetic patients to develop cardiovascular diseases [18–20]. Oxidised LDL is produced in the subendothelial space and is taken up by resident macrophages via scavenger receptors [21–23], leading to their transformation into foam cells. A recent report shows that beta cells express scavenger receptor class B, member 1 and CD36, two scavenger receptors for oxidised LDL, and that incubation of beta cells with oxidised LDL causes a decline in specialised tasks including insulin synthesis [24]. Oxidised LDL can induce oxidative stress in several tissues, including activation of the JNK signalling pathway and AP1 transcriptional activity [25–27]. In view of these data, we postulated that JNK signalling is implicated in the alteration of insulin production and cell survival caused by oxidised LDL.

Herein, we provide evidence that oxidised LDL, but not native LDL, exerts deleterious effects on insulin levels and beta cell survival by activating the JNK pathway. Selective inhibition of this pathway with peptide inhibitors or incubation with human HDLs countered the effects mediated by oxidised LDL.

Materials and methods

Lipoprotein preparation Blood was collected from healthy donors. Plasma LDL fractions were isolated by sequential ultracentrifugation (LDL density, 1.063) and dialysed against PBS. Samples were analysed by SDS-PAGE to assess the integrity of apolipoproteins and the purity of the different fractions. The lipoprotein preparations contained less than 0.112 units of endotoxin/μmol cholesterol as determined by the kinetic chromogenic technique (Endotell, Allschwil, Switzerland). Oxidation of LDL particles (at 1 mg/ml protein concentration in PBS) was performed by incubation with 5 μmol/l $CuSO_4$ at 37°C for 18 h. The oxidation reaction was stopped at 4°C for 30 min by adding 300 μmol/l EDTA and by thorough dialysis against PBS and subsequently against either DMEM or RPMI medium without fetal calf serum. The oxidation reaction was verified by determining the lipid peroxide content as previously described [28].

Preparation and culture of islets Rat islets were isolated from the pancreas of male Sprague–Dawley rats weighing 250–350 g by ductal injection of collagenase. The purification of islets was conducted as described [29]. Isolated human islets were obtained from the Cell Isolation and Transplantation Center (islets for research distribution programme) of the Geneva University Hospitals. Islets were cultured in CMRL-1066 supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA, USA) in a 5% CO_2 humidified atmosphere at 37°C.

Cell culture, transient transfection and plasmids The insulin-secreting cell lines (MIN6 and INS-1E) and rat isolated islets were maintained as previously described [30]. Transient transfection experiments were performed using a kit (Effectene Transfection Reagent kit; Qiagen, Basel, Switzerland) as reported [30]. The following plasmids were used for the transfection assays: a 600-base pair sequence of the rat insulin II promoter (Rip) cloned upstream of the firefly luciferase gene [11]; rat insulin promoter element $(RIPE)_3$Luc [11]; and a luciferase reporter construct (Luc) driven by multimerised AP1 consensus sequences (AP1Luc). The latter two are firefly luciferase reporter constructs corresponding to five copies of the $(RIPE)_3$ binding site (containing the E elements) and to four copies of the canonical AP1-responsive elements inserted upstream of the TATA minimal promoter, respectively. Luciferase activities from the firefly and the renilla from

the pRL-SV40 vector (Promega, Wallisellen, Switzerland) were measured using an assay system (Dual-Luciferase Reporter; Promega).

Measurement of insulin content Cells (5×10^5) were plated in 24-well dishes and cultured in the presence of vehicle, native and oxidised LDL for 72 h. Afterwards, the cells were washed three times with a modified KRB/bicarbonate-HEPES buffer (140 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 1.5 mmol/l $CaCl_2$, 2 mmol/l $NaHCO_3$, 10 mmol/l HEPES, 0.1% bovine serum albumin) containing 2 mmol/l glucose. Insulin contents were extracted with acid/ethanol solution and measured by ELISA (Linco Research, St Charles, MO, USA) as recommended by the manufacturer's protocol.

Apoptosis assay Apoptosis was determined by scoring cells displaying pycnotic nuclei visualised with Hoechst 33342 (Invitrogen, Basel, Switzerland) [28].

Protein kinase assay The preparation of whole-cell protein extracts and the kinase assays were conducted as previously described [8]. Briefly, cell extracts were incubated for 1 h at room temperature with 1 μg glutathione S-transferase (GST)-Jun (amino acids 1–89) and 10 μl glutathione-agarose beads (Sigma-Aldrich, St-Gallen, Switzerland). After several washings, the beads were supplemented with JNK inhibitor or TAT (control) peptides for 20 min [8]. The JNK inhibitor peptides used were the JNK binding domain of IB1, which was coupled covalently to an N-terminal ten-amino acid carrier peptide derived from the HIV-$TAT_{48–57}$ sequence [8]. The retro-inverso D-enantiomer TAT and JNK inhibitor peptides (Auspep PLT, Melbourne, VIC, Australia) [8] were a gift from C. Bonny (S. A. Xigen, Lausanne, Switzerland). Phosphorylation of substrate proteins was examined after overnight exposition of polyacrylamide gels to autoradiography; gel quantifications were accomplished by Phosphor-Imager analysis (Molecular Imager FX; Bio-Rad Laboratories, Basel, Switzerland).

Nuclear protein extracts preparation and electromobility shift assays Nuclear protein extracts and binding reaction were conducted exactly as previously reported [30]. The primers used as labelled probe were: AP1: sense: 5′-CG CTTGATGAGTCAGCCGGAA-3′ and antisense 5′-GGC TGACTCATCAAGCG-3′.

Western blotting, total RNA preparation and real-time PCR For western blotting, the cell extracts were separated by SDS-PAGE and blotted on nitrocellulose membranes. The proteins were detected using specific antibodies and were visualised by chemiluminescence using horseradish peroxidase-coupled secondary antibodies. Total RNA from insulin-secreting cell lines and pancreatic islets was extracted using an RNA purification kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Reverse transcription reactions were performed as previously described [30]. Real-time PCR assays were carried out on a real-time PCR detection system (MyiQ Single-Colour; Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), with 100 nmol/l primers, 1 μl of template per 20 μl of PCR and an annealing temperature of 59°C. Melting curve analyses were performed on all PCRs to rule out non-specific amplification. Reactions were carried out in triplicate. Primer sequences for PCR were as follows: mouse and human insulin, sense 5′-TGGCTTCTTCTACACACCCA-3′, antisense 5′-TCTAGTTGCAGTAGTTCTCCA-3′; mouse and human tubulin, sense 5′-GGAGGATGCTGCCAATAACT-3′, antisense 5′-GGTGGTGAGGATGGAATTGT-3′; rat and mouse *Ib1*, sense 5′-AGTGTCCAGCTTCCCTTGTC-3′, antisense 5′-TTACTGTGGCCCTCTCCTTG-3′; human *IB1*, sense 5′-ATCAGCCTGGAGGAGTTTGA-3′, antisense 5′-AGGTCCATCTGCAGCATCTC-3′; human *FOS*, sense 5′-TGATACACTCCAAGCGGAGAC-3′, antisense 5′-CCC AGTCTGCTGCATAGAAGG-3′; mouse ribosomal phospho-protein P0 gene, sense 5′-ACCTCCTTCTTCCAGGCTTT-3′, antisense 5′-CCACCTTGTCTCCAGTCTTT-3′; mouse B-cell leukaemia/lymphoma 2 (*Bcl2*), sense: 5′-CTCCCGATT CATTGCAAGTT-3′, antisense 5′-TCTACTTCCTCCG CAATGCT-3′.

Statistical analyses Data are expressed as means±SEM. Unpaired two-tailed Student's *t* test was used to compare groups.

Results

Oxidised LDL particles reduce insulin expression at the transcript level We first investigated the effects of oxidised LDL particles on insulin production. In vitro oxidisation of LDL-cholesterol particles by copper has been previously shown to generate similar changes in LDL particles to those occurring in endothelial cells, including lipid peroxidation and extensive hydrolysis of phosphatidylcholine [31]. For this reason, we chose to oxidise LDL particles by copper, as performed in many reports [24, 32, 33], in order to evaluate the effects of oxidised LDL on beta cells. Insulin contents were measured from MIN6 cells cultured with different concentrations of freshly purified oxidised LDL-cholesterol particles. The results show that oxidised LDL reduced insulin content in a dose-dependent manner, whereas native LDL did not affect insulin expression (Fig. 1a). Similar results were obtained in INS-1E cells (data not shown) and are consistent with the data of a previous study showing

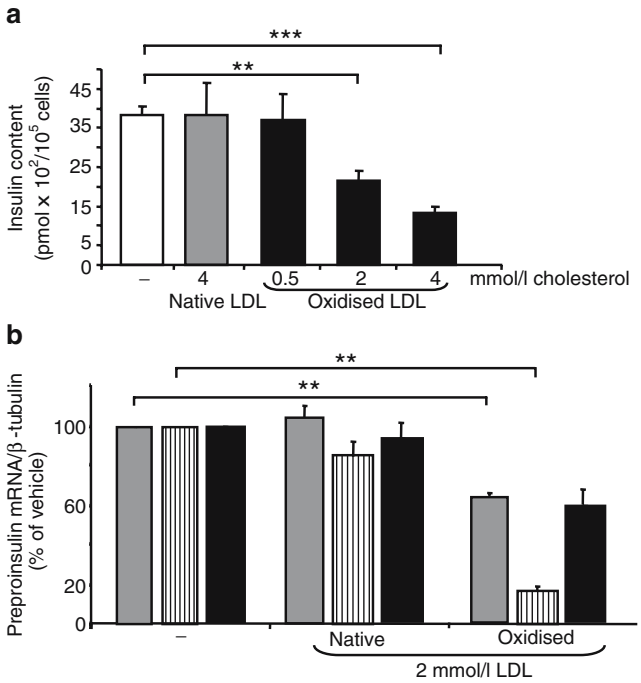

Fig. 1 Effects of oxidised LDL on insulin levels. **a** Insulin content of MIN6 cells exposed for 72 h to vehicle (−), native LDL and different concentrations of oxidised LDL-cholesterol particles. Data (mean ± SEM) are representative of four independent experiments. **b** Analysis of representative insulin mRNA levels by real-time PCR. Total RNA was isolated from MIN6 cells (*grey bars*), isolated rat islets (*striped bars*) and isolated human islets (*black bars*) that had been cultured for 72 h with different LDL preparations. Total RNA was then subjected to real-time PCR to measure the preproinsulin and β-tubulin (internal control) mRNA levels. Data from cells cultured with vehicle (−) were set to 100%. Data are the mean ± SEM of three independent experiments. ** $p<0.01$; *** $p<0.001$

that native LDL at cholesterol concentrations between 1.6 and 3.1 mmol/l has no effect on beta cell survival and function [28]. To evaluate the effects of modified LDL for the following experiments, we chose to incubate the cells at an oxidised LDL concentration of 2 mmol/l. Under these conditions, as previously reported [24], we found that the decrease in insulin content was associated with a loss in preproinsulin mRNA levels (Fig. 1b). These oxidised LDL-mediated effects also occurred at 48 h (data not shown) of culture and were observed in isolated human and rat islets (Fig. 1b), as well as in the insulin-secreting cell lines MIN6 (Fig. 1b) and INS-1 (data not shown).

Oxidised LDL diminishes the expression of insulin at the promoter level We next assessed the hypothesis that oxidised LDL-mediated effects on insulin expression occurs at transcriptional levels. For this purpose, a 600-base pair fragment of rat insulin II promoter (Rip) linked to the luciferase reporter (RipLuc) construct was transiently transfected in INS-1E cells [11]. While culture of the transfected cells with native LDL did not significantly modify the luciferase activity of RipLuc, oxidised LDL caused a drastic decrease in production of the reporter gene (Fig. 2a). The activity of the Rip is dependent for a large part on an enhancer region (5′-GCCATCTG-3′), which is referred to as insulin control element or E element [34, 35]. When multimerised and cloned downstream of a SV40 promoter, this region is capable of enhancing expression of a luciferase gene from the $(RIPE)_3$Luc construct [11, 13]. To verify whether the E element is responsible for the loss of insulin expression, INS-1E cells transfected with this heterologous promoter construct were cultured in the presence of oxidised LDL. We found that incubation with oxidised LDL but not with native LDL generated a twofold decrease in the luciferase activity of $(RIPE)_3$Luc (Fig. 2b).

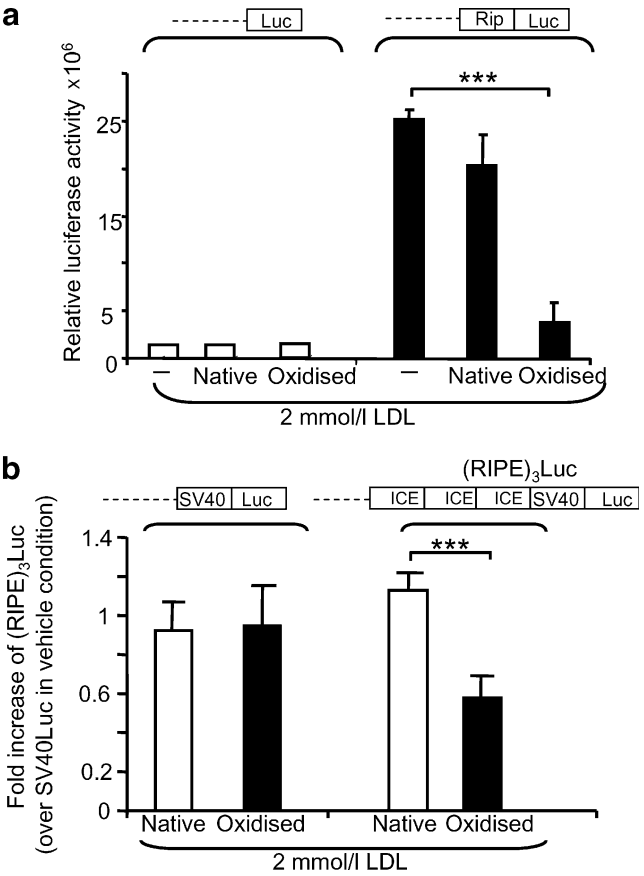

Fig. 2 Effects of oxidised LDL on insulin reporter construct activity in MIN6 cells. Vehicle (−), native and oxidised LDL-cholesterol particle preparations were added to culture medium 2 h after transfection. Exposure of cells to oxidised LDL led to a reduction of (**a**) the luciferase activity of a 600-bp fragment of the rat insulin promoter (RipLuc) and (**b**) the heterologous promoter activity containing insulin control element (ICE) $(RIPE)_3$Luc. The empty pGL3basic (Luc) and the SV40Luc vectors were used as controls for measuring the promoter activity of RipLuc and $(RIPE)_3$Luc, respectively. All luciferase activities were normalised using pRLSV40 renilla. Each experiment was performed at least three times in triplicate. All values are expressed as per cent of SV40Luc activity in cells cultured with vehicle (−). Results are mean ± SEM. *** $p<0.001$

Introduction of a mutation in E elements [11] prevented the loss of luciferase activity mediated by oxidised LDL (data not shown). This indicates that the E element is responsible for the loss of insulin expression.

Oxidised LDL induces JNK activity, increases AP1 transcriptional activity and downregulates expression of Ib1 It is well documented that the E element of Rip binds the basic helix loop helix transcription factors [34, 35]. Previous reports have demonstrated that c-Jun, a component of the AP1 transcriptional complex [36], can inhibit the E47 basic helix loop helix factor, leading to inhibition of insulin promoter activity [13, 14]. Interestingly, oxidised LDL led to oxidative stress in various cell types [25, 27]. This phenomenon involves activation of the JNK signalling cascade and culminates in an increase in the activity of AP1 transcriptional complexes due to an elevation of c-Jun levels and its activity [25, 37, 38]. Based on these observations, we hypothesised a possible increase in AP1 activity in cells cultured with oxidised LDL. To test this assumption, we monitored AP1 activity by transiently transfecting a luciferase reporter construct driven by multimerised AP1 consensus sequences (AP1Luc). While no changes in AP1Luc activity were observed in the presence of native LDL, in cells cultured with 2 mmol/l oxidised LDL-cholesterol a twofold increase in the luciferase activity of AP1Luc was detected (Fig. 3). In addition, electromobility shift assay experiments performed using the AP1 consensus sequence as labelled probe revealed an increase in AP1 binding pattern in nuclear extracts from cells cultured with oxidised LDL (Electronic supplementary material [ESM] Fig. 1).

To determine whether the rise in AP1 activity was the result of increased JNK activity, we measured JNK-mediated phosphorylation of the target transcription factor c-Jun. Total proteins from cells cultured with either 2 mmol/l of oxidised LDL or native LDL were incubated with the c-Jun recombinant. Using extracts of cells treated by oxidised LDL, kinase experiments show a time-dependent increase in c-Jun phosphorylation (Fig. 4a,b).

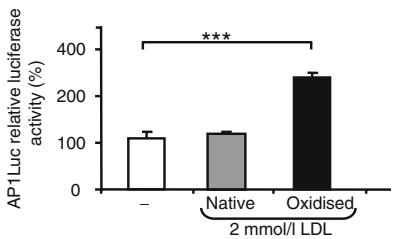

Fig. 3 Assessment of AP1 transcriptional activity in cells challenged with oxidised LDL. MIN6 cells were transiently transfected with a luciferase reporter construct driven by multimerised AP1 consensus sequences (AP1Luc). Data are expressed as per cent of control (activity of the construct in cells incubated with vehicle) and are the mean ± SEM of three independent experiments. *** $p<0.001$

As expected, the phosphorylation of c-Jun was efficiently blocked by 5 μmol/l JNK inhibitor peptides [8] or by the selective JNK inhibitor SP600125 (ESM Fig. 2). Phosphorylation of c-Jun occurred with extracts of cells incubated for 45 min and 72 h with oxidised LDL, whereas native LDL did not induce any change in phosphorylation of c-Jun. Phosphorylation of JNK is required for JNK to

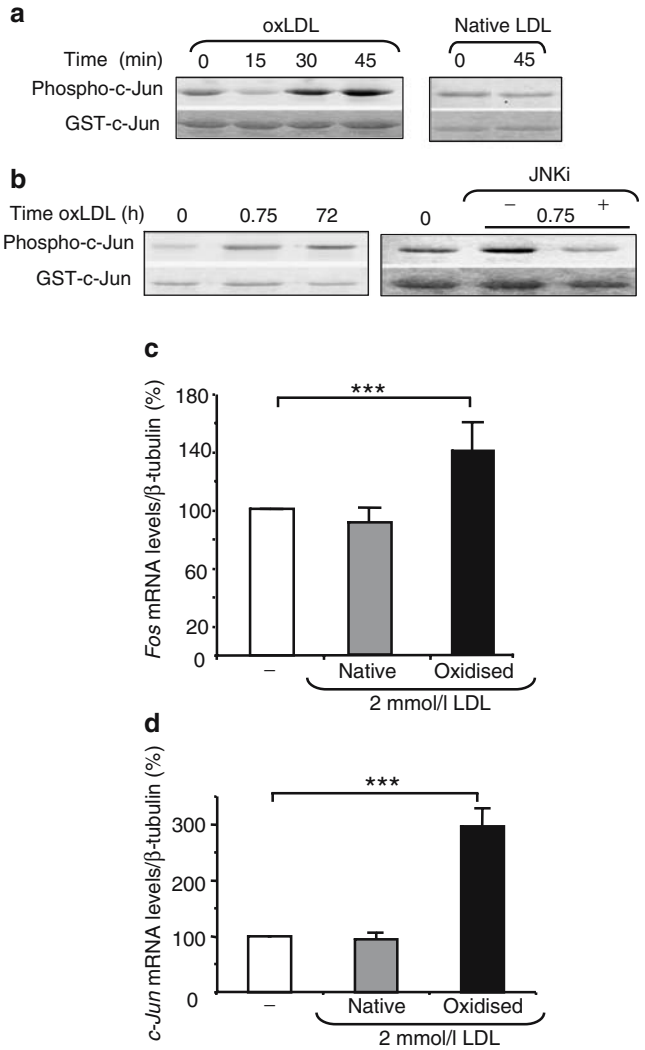

Fig. 4 Effects of oxidised LDL (oxLDL) on JNK activity. **a**, **b** Whole-cell extracts were prepared from cells incubated with 2 mmol/l oxidised LDL or native LDL at the indicated times. JNK inhibitor peptides (JNKi) (**b**), at a 5 μmol/l concentration, were added in cells cultured with oxidised LDL (2 mmol/l). JNK solid-phase JNK assays were performed with the lysates using GST-c-Jun as substrate. The reaction was loaded on a polyacrylamide gel and γ-^{33}P-phosphorylation of the substrates (Phospho-c-Jun) was subsequently analysed. The gel was stained with Coomassie blue to evaluate the loading of substrate (GST-Jun). The results are representative of three independent experiments. **c**, **d** Measurement of *c-Jun* and *Fos* expression levels by real-time PCR. The mRNA levels of these genes were normalised against β-tubulin and expression levels from cells cultured with vehicle were set to 100%. Data are the mean ± SEM of four independent experiments. *** $p<0.001$

phosphorylate its substrates. Consistent with this, western blotting experiments showed an increase in JNK activity in cells exposed to oxidised LDL (Fig. 4a,b). Thus, these results confirm that activation of JNK is induced by oxidised LDL. To validate activation of the JNK signalling cascade by oxidised LDL, expression of the *c-Jun* and *Fos* genes was then quantified. The transcriptional activity of the promoters of these two genes is positively regulated by JNK [8]. Real-time PCR analysis showed a statistically significant augmentation by 1.5- and threefold in *Fos* and *c-Jun* mRNA levels, respectively, in MIN6 cells cultured with oxidised LDL (Fig. 4c,d).

We next investigated whether JNK activation is responsible for the loss of insulin production. Insulin-secreting cells were incubated with oxidised LDL in the presence of JNK inhibitor. Treatment of the cells with these peptides prevented the decrease in preproinsulin mRNA and $(RIPE)_3$Luc promoter activity caused by oxidised LDL (Fig. 5a,b). Prolonged exposure of cells to various stressors has been shown to activate the JNK pathway in beta cells. In some cases, this activation results from the decline of IB1 levels [15, 16, 39]. This prompted us to evaluate the expression of *Ib1* in cells treated with the oxidised LDL preparation. Western blotting showed a decrease in IB1 protein contents in MIN6 cells treated with oxidised LDL for 72 h, whereas the content was unaffected at 45 min of culture (Fig. 6a). Real-time PCR confirmed the reduction in *IB1* mRNA levels in isolated human and rat islets as well as in MIN6 cells cultured with oxidised LDL for 72 h (ESM Fig. 3).

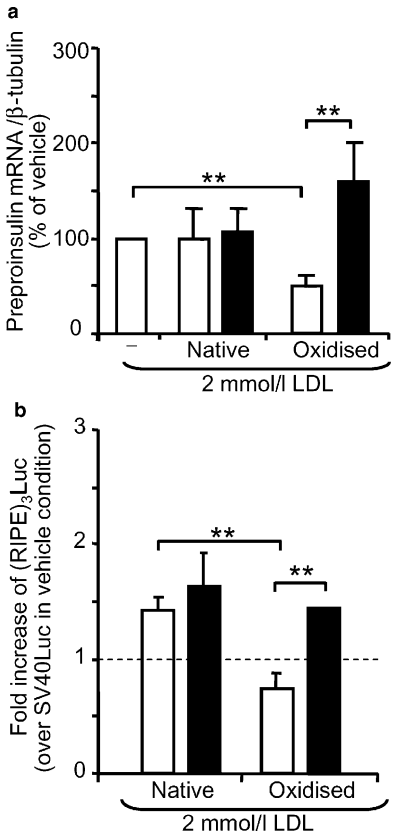

Fig. 5 Effects of JNK inhibition on the loss of insulin expression mediated by oxidised LDL. **a** JNK inhibitor (*filled bars*) or D-TAT (*open bars*) as control [8] was co-incubated with the different LDL preparations in MIN6 cells and preproinsulin mRNA levels were quantified by real-time PCR experiments. Data are the mean ± SEM of four independent experiments. **b** Effects of JNK inhibitor on the activity of the $(RIPE)_3$Luc construct. MIN6 cells were transiently transfected with $(RIPE)_3$Luc and co-cultured with LDL preparations and 5 μmol/l of the JNK inhibitor (*filled bars*) or TAT (*open bars*) for 48 h. Luciferase activities were normalised using pRLSV40 renilla. Each experiment was performed at least three times in triplicate. All values are expressed as per cent of SV40Luc activity. Results are expressed as mean ± SEM. ** $p<0.01$

Activation of the JNK pathway has often been associated with an increase in beta cell programmed death [8, 15, 40]. We therefore tested the viability of insulin-secreting cells in the presence of LDL-cholesterol preparations. As expected, the rate of apoptosis of the cells cultured with 2 mmol/l oxidised LDL-cholesterol for 72 h increased by threefold in MIN6 cells and rat isolated islets, whereas the viability of the cells incubated in the presence of native LDL was unchanged (Fig. 6b). The rate of apoptosis was similar when MIN6 cells were incubated either at 20 or at 10 mmol/l glucose. This result is in agreement with a previous report showing that glucose did not render cells more sensitive to the effects of LDL [28]. Co-treatment with JNK inhibitors prevented the apoptosis of cells mediated by oxidised LDL (Fig. 6c). Real-time PCR experiments showed a reduction of *Bcl2* expression (Fig. 6d). This result is in agreement with the induction of the apoptotic pathway by oxidised LDL as reported in endothelial cells [41]. In contrast to early effects of oxidised LDL on insulin levels, the viability of the cells cultured with oxidised LDL for 48 h was apparently unchanged (data not shown). This result indicates that the loss of insulin expression induced by oxidised LDL probably precedes programmed cell death.

Taken together, our results indicate that the effects of oxidised LDL on insulin expression and beta cell survival are linked to activation of the JNK signalling pathway resulting from a decrease in IB1 contents.

HDL protects cells from oxidised LDL-induced loss of insulin expression and death HDL has been described to protect beta cells from cytokine- and LDL-mediated apoptosis [28]. To assess the potential protective effects of HDL on the decline of insulin expression and the induction of cell death mediated by oxidised LDL, MIN6 cells and isolated human islets were co-incubated with HDL and LDL preparations. HDL at 1 mmol/l of cholesterol concentration protected the cells from apoptosis (Fig. 7a)

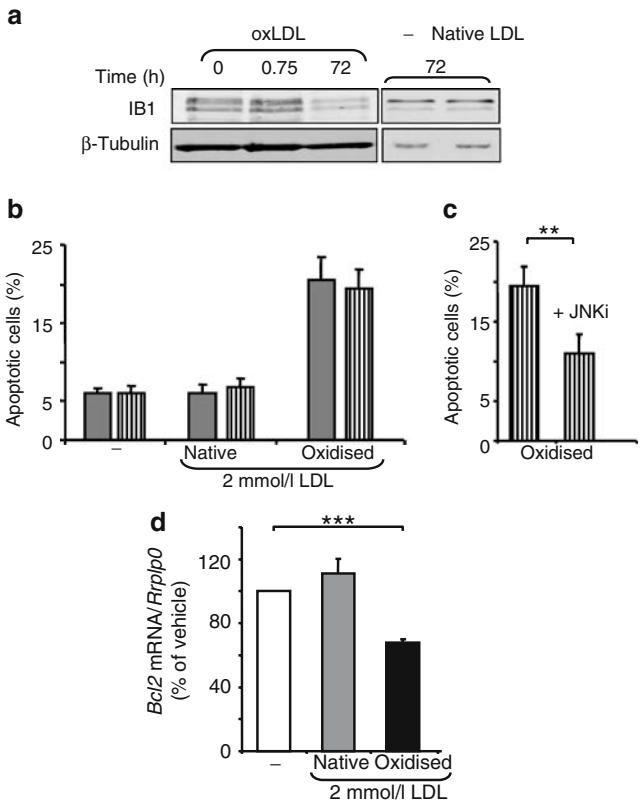

Fig. 6 Analysis of IB1 levels. **a** The levels of IB1 in MIN6 cells cultured at indicated times with 2 mmol/l LDL-cholesterol preparation or vehicle (−) were assessed by western blotting. The results are representative of three independent experiments. **b**, **c** The rate of apoptosis was scored in MIN6 cells (*grey bars*) or isolated rat islets (*striped bars*), co-cultured with 2 mmol/l LDL preparation (oxidised or native) or vehicle (−), in the presence (**c**) or absence of JNK inhibitor (JNKi). Each experiment was performed three times in triplicate. Results are expressed as mean ± SEM. ** $p<0.01$. **d** Expression of *Bcl2* was quantified by quantitative PCR. The mRNA level was normalised against the housekeeping acidic ribosomal phosphoprotein P0 gene (*Rplp0*) and expression levels from cells cultured with vehicle were set to 100%. Data are the mean ± SEM of three independent experiments. *** $p<0.001$

and prevented the reduction in *Bcl2* expression induced by oxidised LDL (Fig. 7b). In addition, HDL treatment partially prevented the loss of preproinsulin mRNA and promoter activity (Fig. 8a,b). The protective effects of HDL on the activity of RipLuc were also observed at 48 h of incubation (data not shown). HDL also efficiently prevented oxidised LDL-induced AP1Luc activity (Fig. 9a) and an increase in *Fos* expression in MIN6 cells (Fig. 9b) and human isolated islets (Fig. 9c). The restoration of basal activity of AP1 by HDL was already observable at 48 h (data not shown). In line with the loss of AP1 activity, HDL countered JNK's effects on c-Jun phosphorylation (Fig. 9d) and the decline in *IB1* expression (Fig. 9e,f). These data suggest that HDL exerts its protective action by blocking the effects of oxidised LDL on the JNK signalling pathway.

Discussion

Several studies have reported expression of receptors for native and modified forms of LDL, including scavenger receptor class B, member 1 and CD36 scavenger receptors, as well as the uptake of these lipoproteins in pancreatic beta cells [24, 28, 33, 42]. Herein, in agreement with a previous study, we found that insulin-secreting cells cultured with oxidised LDL have reduced preproinsulin gene expression [24]. This perturbed expression was observed at protein and mRNA levels, both in isolated human and rat islets and in several insulin-secreting cell lines. The reduced luciferase activity of RipLuc and the multimerised E elements-containing promoter $(RIPE)_3$Luc suggests that oxidised LDL particles exert their action on the preproinsulin gene at the transcriptional levels. This effect is mediated through the E elements and is not the result of irreversible cell damage as can be seen from the fact that mutation of these elements prevents the changes in luciferase activity of $(RIPE)_3$Luc triggered by oxidised LDL. Several lines of evidence support a role for c-Jun, a component of the AP1 transcriptional complex, in the decline of preproinsulin gene transcription mediated by oxidised LDL [13]. Overexpression of *c-Jun* has been shown to indirectly repress the enhancer activity of the E elements by interfering with the transactivating capacity of basic helix loop helix transcription factors, which bind to the former element [13, 14]. Conversely, induction of insulin expression is accompanied by a decrease in *c-Jun* expression [43]. In line with these observations, we found that the impaired preproinsulin expression is associated with an increase in AP1 transcriptional activity and *c-Jun* expression.

AP1 activity and *c-Jun* expression are induced by an unusually broad range of environmental stressors [44]. Many of these stimuli activate JNKs, leading to enhanced AP1 transcriptional activity. In cultured human fibroblasts and human aortic endothelial cells, oxidised LDL has been reported to increase the activity of AP1 and JNK, respectively [25, 26, 45]. Moreover, activation of the JNK pathway by overexpressing mitogen activated protein kinase kinase kinase 1 in insulin-secreting cells reduces the luciferase activity of $(RIPE)_3$Luc [11]. For this reason, we presumed that activation of JNK by oxidised LDL was responsible for augmenting AP1 activity and therefore for reducing preproinsulin gene expression. Consistent with this, inhibition of JNK with JNK inhibitors prevented the loss of $(RIPE)_3$Luc activity and the decrease in preproinsulin mRNA levels mediated by oxidised LDL. This finding furnishes new evidence for the possible involvement of the JNK pathway in impaired preproinsulin expression through the E element.

Increased JNK activity and *c-Jun* expression have been reported in many scenarios in which cells undergo

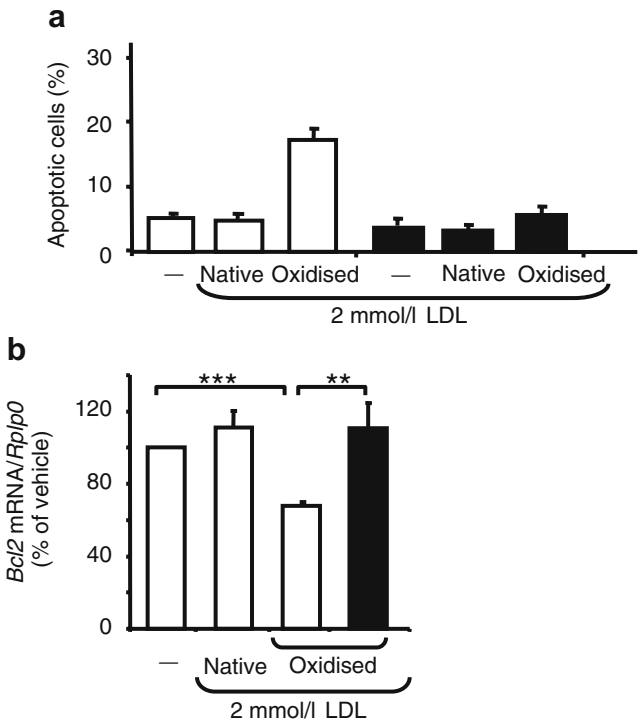

Fig. 7 Protective effects of HDL against apoptosis mediated by oxidised LDL. **a** Apoptosis was scored in MIN6 cells that were co-cultured in the presence of different LDL preparations for 72 h with 1 mmol/l HDL-cholesterol (*filled bar*) or without (*open bars*). HDL LDL **b** The mRNA level of *Bcl2* was quantified by real-time PCR in MIN6 cells cultured with oxidised LDL and with (*filled bar*) or without (*open bars*) 1 mmol/l HDL. The mRNA level was normalised against β-tubulin and the expression levels from cells cultured with vehicle were set to 100%. Data are the mean ± SEM of three independent experiments. ** $p<0.01$, *** $p<0.001$

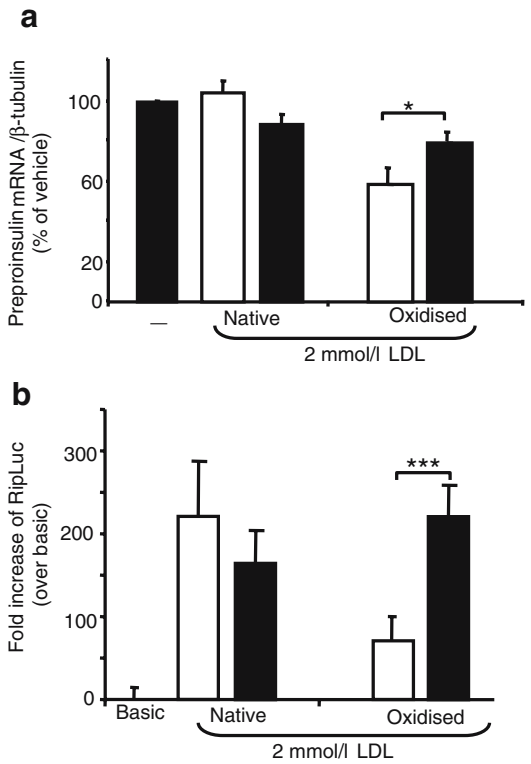

Fig. 8 Protective effects of HDL on the loss of insulin levels induced by oxidised LDL. **a** The preproinsulin mRNA levels were quantified by real-time PCR experiments in cells incubated for 72 h in the presence (*filled bars*) or absence (*open bars*) of 1 mmol/l of HDL and 2 mmol/l of LDL preparation. Results are expressed as the mean ± SEM of at least three independent experiments measured in triplicate. * $p<0.05$. **b** Transient transfection experiments were performed to monitor the activity of RipLuc in cells incubated for 72 h in the presence (*closed bars*) or absence (*open bars*) of 1 mmol/l of HDL and 2 mmol/l of LDL preparation. Data (**b**) are expressed as fold increase of RipLuc over the pGL3basic vector (Basic). All results are expressed as the mean ± SEM of at least three independent experiments measured in triplicate. *** $p<0.001$

apoptosis [46, 47]. As expected, oxidised LDL induced a rise in the rate of apoptosis and JNK inhibitors protected cells against this deleterious effect. The reduction of *Bcl2* expression confirms activation of the apoptotic pathway induced by oxidised LDL. In contrast, native LDL at 2 mmol/l cholesterol concentration did not induce apoptosis. This result is in agreement with previous studies showing that native LDL-cholesterol at concentrations lower than 3.1 mmol/l cholesterol does not cause apoptosis [24, 28]. Some reports found no effects of oxidised LDL on apoptosis [24, 33]. The discrepancy between the latter studies and our data could be due to the length of exposure to oxidised LDL. Indeed, we observed that 48 h incubation with oxidised LDL is sufficient to initiate a slight decrease in insulin levels but not to affect the rate of apoptosis (data not shown). Thus, these data confirm that the loss of insulin levels caused by oxidised LDL is not a consequence of cell death and might precede the apoptotic events.

IB1 is a key modulator of the JNK pathway and is required for cell survival and insulin expression [11, 15]. Long-term exposure of cells to proapoptotic stimuli diminishes expression of *Ib1* [15, 16]. Such a decrease induces the JNK pathway and the subsequent activation of AP1, thereby leading to impaired insulin synthesis and increased apoptosis [11, 15, 16]. As expected, IB1 levels were reduced in cells treated with oxidised LDL for 72 h. In contrast, these levels were unchanged after 45 min of treatment, although JNK activity occurred at that time point. Therefore, the data show that the early effects of oxidised LDL on induction of JNK activity do not require downregulation of IB1. However, the latter event can be responsible for maintaining prolonged activation of JNK.

In patients with diabetes and metabolic syndrome, low serum HDL levels and elevated oxidised LDL concentrations are risk factors for the the development of cardiovascular diseases [18–20]. While oxidised LDL has pro-atherogenic effects, HDL is known to be anti-athero-

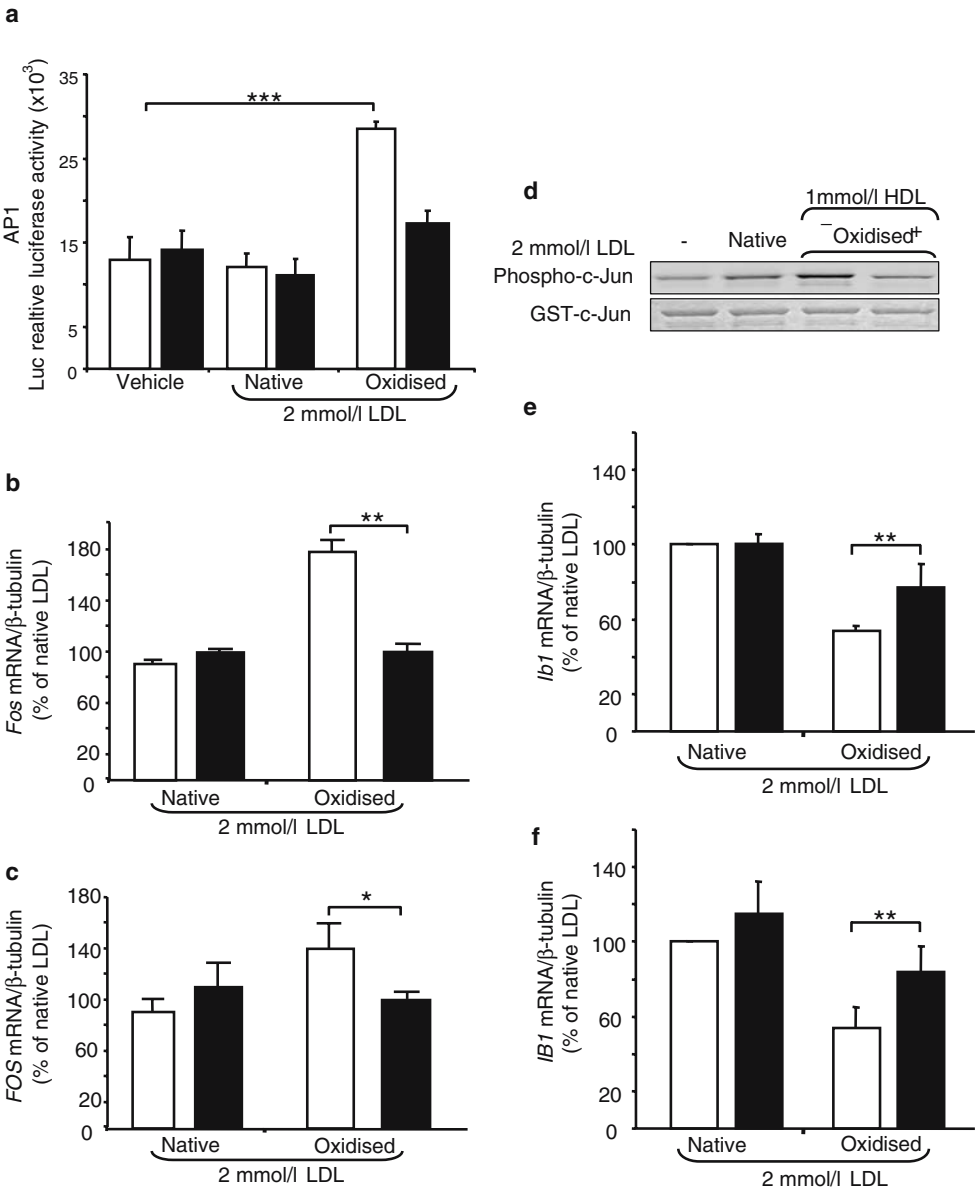

Fig. 9 HDL prevents induction of the JNK pathway mediated by oxidised LDL. **a** MIN6 cells were transiently transfected with the AP1Luc construct and incubated with vehicle, native and oxidised LDL with (*closed bars*) or without (*open bars*) 1 mmol/l HDL. **b** *Fos* mRNA levels were measured in MIN6 cells and (**c**) in human islets co-cultured with LDL preparations and with (*filled bars*) or without (*open bars*) 1 mmol/l HDL-cholesterol for 72 h. **d** JNK activity was measured in whole-protein extracts from MIN6 cells cultured with vehicle (−), native and oxidised LDL plus or minus 1 mmol/l HDL. **e** The effects of HDL on *Ib1* mRNA levels were assessed in MIN6 cells and **f** in human isolated islets exposed to LDL preparations and HDL (*filled bars*) for 72 h. The mRNA level was normalised against β-tubulin. Expression levels from cells cultured with native LDL were set to 100%. Data in all panels are the mean ± SEM of five independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

genic and cardioprotective. Besides its role in the reverse transport of cholesterol, HDL has been shown in vitro to exert its effects by inhibiting LDL oxidation and cell signalling mediated by oxidised LDL; it also counters several adverse biological effects, such as cytotoxicity and inflammatory responses triggered by oxidised LDL [19, 48, 49]. In this report, we establish that HDL efficiently counters the effects of oxidised LDL on apoptosis by restoring expression of *Bcl2*. One possible mechanism is the depletion by HDL of LDL from lipid peroxides through enzymatic hydrolysis of phospholipid hydroperoxides, a process effected by the HDL-bound enzyme paraoxonase. This has been shown to reduce cytokine production stimulated by oxidised LDL [50]. In addition, the idea that HDL mediates its action by preventing activation of the JNK pathway is supported by the fact that JNK and AP1 activities were not induced and that IB1 and FOS levels were unaltered in cells co-cultured with oxidised LDL and HDL. This hypothesis is supported by the fact that HDL protects insulin-secreting cells from apoptosis induced by VLDL [28]. As is the case here for oxidised LDL, VLDL-mediated apoptosis was linked to impaired *Ib1* expression and an increase in JNK activity [28].

Our data also show that HDL exerted its effects on insulin levels and AP1 activity as early as the 48-h time point. At that incubation time, downregulation of *Ib1* and apoptosis did not occur. Thus, HDL counters the oxidised LDL-mediated activation of JNK in a manner that is both dependent and independent of IB1. More than 20 proteins are associated with HDL. These include apolipoproteins that serve as

structural components, cofactors or inhibitors of enzymes, as well as ligands of receptors. Future studies will have to clarify which of these components mediate the protective effects of HDL, which counter those of oxidised LDL, on beta cells.

Finally, this study highlights the biological consequences and the relationship between the levels of the modified lipoproteins and HDL for beta cell function. Reductions in the concentration of HDL could potentiate the effects of oxidised LDL, thereby contributing to beta cell dysfunction. Therefore, like hyperglycaemia and NEFA, modified LDL could contribute to the development of diabetes. A better understanding of the mechanisms underlying the effects of oxidised LDL and HDL will help elucidate the causes of human type 2 diabetes and may lead to novel strategies for treatment or prevention of diabetes.

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B Oxidative stress and ICER mediate the harmful effects of oxLDL on β-cell function

B Oxidative stress and ICER mediate the harmful effects of oxLDL on β-cell function

B.1 Introduction

Pancreatic β-cell dysfunction represents a key step in the pathogenesis of T2D, as this process underlies the transition between an asymptomatic state of β-cell compensation and overt diabetes. This alteration is characterized by impairment in the biosynthesis and secretory capacities of β-cells and exacerbated rate of apoptosis. The chronic exposure of pancreatic β-cells to excess of metabolic fuels, such as FFA and glucose, contributes to the progressive decline in cellular function and glucose homeostasis (14). These environmental stressors chronically elevate intracellular ROS which interfere with the proper function of β-cells – a process termed oxidative stress (44). As previously mentioned, hyperglycemia and hyperlipidemia stimulate the expression of the transcriptional repressor ICER, thereby promoting β-cell dysfunction (71;73;82).

Patients with T2D and glucose intolerance have a higher risk to develop atherosclerosis and associated cardiovascular diseases, as a consequence of the elevation in the circulating levels of oxidatively modified over native LDL and the reduced concentrations of HDL. Since these perturbations in lipid profile occur long before the establishment of frank hyperglycemia, a role for these lipoproteins in the pathogenesis of T2D and particularly β-cell dysfunction has been postulated. Several data, including the results of the precedent chapter, support the idea that oxidized lipoproteins together with low levels of HDL contribute to the impairment in insulin biosynthesis and release, and β-cell survival (56;80). These perturbations ensue from the activation of the JNK pathway, which is one of the hallmarks of oxidative stress, as demonstrated in the previous chapter.

These observations led us to hypothesize that oxidized LDL may trigger β-cell failure by inducing the transcriptional repressor ICER in a mechanism that involves oxidative stress.

B.2 Results

In order to study the molecular mechanism underlying the adverse outcomes of oxidized lipoproteins, we first determined the expression of the transcription factor ICER in pancreatic β-cells exposed for 72h in presence of native or oxidized human LDL. Whereas native lipoproteins had no effect, modified LDL significantly increased ICER mRNA and protein levels in the insulin-secreting cell lines MIN6 B1 and INS-1E, as well as in rat isolated islets (Fig 1). The activation of this transcriptional repressor was associated with impairment in the expression of the ICER target genes Rab3a, Rab27a, Slp4, Noc2 and IB1, which was significantly restored by the silencing of ICER (Fig 2b-f). Importantly, the invalidation of ICER expression was correlated with a protection of insulin gene transcription, secretory activity and β-cell survival in presence of oxLDL (Fig 2a and 3).

Induction of the transcriptional repressor ICER by modified lipoproteins was paralleled by the generation of oxidative stress, as demonstrated by the amplified production of ROS (Fig 4a). Oxidative stress proved to be causal in the activation of ICER by oxLDL since the direct ROS generator hydrogen peroxide activated expression of the transcriptional repressor, whereas the antioxidants N-acetylcysteine (NAC) or HDL particles inhibited ICER induction by modified lipoproteins (Fig 4b-e and 6). In agreement with the inhibitory properties of NAC on ICER, antioxidant treatment of pancreatic β-cells fully protected insulin gene expression, insulin secretion and cell survival against the harmful effects of oxLDL (Fig 5).

In conclusion, all these data demonstrate that oxidized LDL contribute to β-cell dysfunction by activating the transcriptional repressor ICER in a mechanism involving oxidative stress.

B.3 Contribution

In this paper, the student was the principal investigator and performed all the experiments. He was helped by his colleagues to increase the number of experimenters on key experiments and to assess the reproducibility of the results. The student performed the preparation of the figures, the statistical analysis of the data and contributed to the redaction of the manuscript. Finally, he designed and made the experiments for the correction of the paper with the help of his colleagues.

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ARTICLE

Role for inducible cAMP early repressor in promoting pancreatic beta cell dysfunction evoked by oxidative stress in human and rat islets

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Abstract
Aims/hypothesis Pro-atherogenic and pro-oxidant, oxidised LDL trigger adverse effects on pancreatic beta cells, possibly contributing to diabetes progression. Because oxidised LDL diminish the expression of genes regulated by the inducible cAMP early repressor (ICER), we investigated the involvement of this transcription factor and of oxidative stress in beta cell failure elicited by oxidised LDL.
Methods Isolated human and rat islets, and insulin-secreting cells were cultured with human native or oxidised LDL or with hydrogen peroxide. The expression of genes was determined by quantitative real-time PCR and western blotting. Insulin secretion was monitored by EIA kit. Cell apoptosis was determined by scoring cells displaying pycnotic nuclei.
Results Exposure of beta cell lines and islets to oxidised LDL, but not to native LDL raised the abundance of ICER. Induction of this repressor by the modified LDL compromised the expression of important beta cell genes, including insulin and anti-apoptotic islet brain 1, as well as of genes coding for key components of the secretory machinery. This led to hampering of insulin production and secretion, and of cell survival. Silencing of this transcription factor by RNA interference restored the expression of its target genes and alleviated beta cell dysfunction and death triggered by oxidised LDL. Induction of ICER was stimulated by oxidative stress, whereas antioxidant treatment with *N*-acetylcysteine or HDL prevented the rise of ICER elicited by oxidised LDL and restored beta cell functions.
Conclusions/interpretation Induction of ICER links oxidative stress to beta cell failure caused by oxidised LDL and can be effectively abrogated by antioxidant treatment.

Keywords Antioxidant · Apoptosis · Diabetes · HDL · ICER · Insulin · Oxidative stress · Oxidised LDL · Pancreatic beta cells

Abbreviations

CRE	cAMP responsive elements
CREluc	Luciferase reporter construct driven by two CRE sequences linked upstream to a SV40 promoter
hGH	Human growth hormone
ICER	Inducible cAMP early repressor
JNK	c-Jun amino terminal kinase
MAPK	Mitogen-activated protein kinase
NAC	*N*-Acetylcysteine
ROS	Reactive oxygen species
si	Small interfering

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Introduction

A decline in the number of insulin-producing beta cells and/or their intrinsic ability to produce and/or secrete insulin contributes to the pathophysiology of type 2 diabetes [1]. Chronic excess of cholesterol and metabolic fuels, such as the NEFA palmitate and glucose, conspire with genetic factors to trigger beta cell dysfunction and destruction over time [2–6]. Low plasma levels of HDL together with increased levels of oxidised LDL-cholesterol are associated with diabetes, thereby elevating risk of developing atherosclerosis and ultimately cardiovascular diseases [7, 8]. The fact that perturbations of these lipoproteins precede development of diabetes has led to the hypothesis that they could contribute to the progression of the disease [9–12]. So far, numerous clues support a role for oxidised LDL in triggering adverse effects in beta cells and thereby contributing to the pathophysiology of diabetes. Prolonged exposure of insulin-producing cell lines, as well as isolated human and rodents islets to oxidised LDL at physiological cholesterol concentration compromised insulin production and secretion, and increased the rates of apoptosis [6, 13–15], while native LDL at a similar cholesterol concentration did not exert such adverse effects. Co-incubation of cells with HDL permitted abrogation of the harmful effects of oxidised LDL [13]. Positive effects, as well as protective properties of this particle against toxicity generated by chronic hyperglycaemia have also been shown [15, 16], validating the concept that adequate levels of HDL are required for beta cell function and control of glucose homeostasis.

A wealth of data point to oxidative stress as the link coupling oxidised LDL to cell dysfunction and death [17, 18]. The hypothesis that oxidative stress can mediate beta cell dysfunction caused by pro-atherogenic oxidised LDL is further supported by a clinical trial investigating the efficiency of an antioxidant in cardiovascular events in patients with acute coronary diseases [19]. Daily administration of the antioxidant molecule markedly reduced the occurrence of new-onset diabetes [19]. Furthermore, in vitro induction of specific signalling cascades supports involvement of oxidative stress in beta cell failure evoked by oxidised LDL. Activation of the mitogen-activated protein kinase (MAPK) pathways, including MAPK8 (also called c-Jun amino terminal kinase [JNK]), is one of the hallmarks of oxidative stress [20, 21]. Activation of JNK signalling in beta cells leads to programmed cell death and impaired insulin expression [20, 21]. Induction of JNK activity has been demonstrated to contribute to beta cell dysfunction caused by oxidised LDL [13].

Increased JNK activity caused by oxidised LDL results from reduced levels of MAPK8 interacting protein-1 (also called JNK interacting protein 1 or islet brain 1), a potent inhibitor of JNK activity in beta cells [13, 22]. Islet brain 1 is a target of the inducible cAMP early repressor (ICER), a member of the cAMP responsive element (CRE) modulator transcription factors [23]. ICER is a powerful passive repressor, which silences expression of genes that contain a CRE within their promoters [24]. Induction of ICER is in part responsible for beta cell failure evoked by hyperglycaemia and fatty acids [25, 26]. This negative role is achieved by silencing expression of insulin [27] and components of the secretory machinery, such as the GTPases *Rab3a* and *Rab27a*, and their effectors *Slp4* (also known as *Sytl4*) and *Noc2* (also known as *Rph3al*) [25]. All these observations led us to investigate whether oxidised LDL may trigger beta cell failure by inducing production of ICER in a mechanism that involves oxidative stress.

Methods

Lipoprotein preparation Blood was collected from healthy donors. Plasma LDL fractions were isolated by sequential ultracentrifugation (LDL density 1.063) exactly as described [13]. Oxidation of LDL particles was done by incubating 1 mg LDL protein/ml PBS with 5 μmol/l CuSO4 at 37°C for 6–8 h [13]. The oxidation reaction was verified as previously described by determining the lipid peroxide content [13].

Cell culture and preparation of isolated islets The insulin-secreting cell line (MIN6) was maintained as previously described [13]. Isolated human islets were obtained from the Cell Isolation and Transplantation Center (islets for research distribution programme) of the Geneva University Hospital (Geneva, Switzerland). Investigations were been approved by the responsible ethics committee. Islets were cultured in CMRL-1066 supplemented with 10% (vol./vol.) fetal bovine serum (Mediatech, Herndon, VA, USA) in 5% CO_2 humidified atmosphere at 37°C. Rat islets were isolated from the pancreas of male Sprague–Dawley rats weighing 250–350 g by ductal injection of collagenase P (Roche Diagnostics, Rotkreuz, Switzerland). Purification and culture of islets was conducted as described [13]. Experimental protocols were approved by the Cantonal Veterinary Office.

Small interfering RNA and transfection A pool of two 19-nucleotide pre-validated small interfering (si) RNA duplexes that target the mouse and rat *Icer* (also known as *Crem*)

sequence (si-*Icer*) were chemically synthesised by Mycrosynth (Balgach, Switzerland). The two target sequences of *Icer* were: 5′-CTGGAGATGAAACTGCTGC-3′ and 5′-CTGGAGATGAAACTGATGA-3′. Transient transfection experiments were performed using a kit (Lipofectamine 2000 transfection; Invitrogen, Basel, Switzerland) as reported [23].

Measurement of secretion Cells (5×10^5) were plated in 24-well dishes and cultured in the presence of vehicle, native and oxidised LDL for 72 h. When human growth hormone (hGH) was used as a reporter for regulated secretion, the cells were first transiently transfected with a construct encoding the hGH and 24 h later cultured with lipoproteins for 72 h. Thereafter, the cells were washed three times with modified KRB HEPES buffer (10 mmol/l HEPES, 0.1% [wt/vol.] bovine serum albumin) containing insulin secretagogues (20 mmol/l of glucose or 30 mmol/l KCl). Insulin released in the supernatant fraction and insulin cellular contents were measured by EIA (Cayman Chemical, Ann Arbor, MI, USA) as recommended by the manufacturer's protocol. Exocytosis from transfected cells was assessed by ELISA (Roche Diagnostics, Rotkreuz, Switzerland) that measured the amount of hGH released into the medium during the incubation period.

Apoptosis assay Apoptosis was determined by scoring cells displaying pycnotic nuclei (visualised with Hoechst 33342) [13]. The counting was performed blind by three different experimenters.

Measurement of superoxide After treatment, MIN6 cells were washed twice with PBS 1× and scraped in PBS 1×. Cells were dispersed by pipetting and then 10 μmol/l lucigenin and 0.2 mmol/l NADPH were added to the cell suspension. Emitted luminescence was quantified for 12 s in a luminometer (Berthold, Bad Wildbad, Germany). Results were normalised to the DNA content measured using Hoechst 33258 dye (Sigma-Aldrich Chemie, Buchs, Switzerland).

Western blotting, total RNA preparation and real-time PCR For western blotting, nuclear extracts were separated by SDS-PAGE and blotted on nitrocellulose membranes as described [13]. The proteins were detected using specific antibodies and visualised with IRDye 800 (Rockland, Gilbertsville, PA, USA) as secondary antibodies, and quantified in an imaging system (Odyssey Infrared; Li-COR, Lincoln, NE, USA). Total RNA from insulin-secreting cell lines and pancreatic islets was extracted using phenol/chloroform extractions as described previously [13]. Reverse transcription reactions were performed as previously described [23]. Real-time PCR assays were carried out on a real-time PCR detection system (MyiQ Single-Color; Bio-Rad Laboratories, Hercules, CA, USA) using the iQ SYBR Green Supermix (Bio-Rad Laboratories) exactly as previously described by Abderrahmani et al. [13]. Primer sequences of rat/mouse origin for *Rab3a*, *Slp4*, *Icer* and *Rplp0* were those published elsewhere [25]. Otherwise primer sequences are described in the electronic supplementary material [ESM] Table 1.

Statistical analyses Experiments including more than two groups were analysed by ANOVA or with the non-parametric equivalent Kruskal–Wallis test.

Results

Oxidised LDL-cholesterol particles hamper insulin production and insulin secretion, as well as beta cell survival by inducing the activity of Icer We first assessed whether induction of *Icer* links oxidised LDL to beta cell failure. In vitro oxidisation of LDL-cholesterol particles by copper have been previously shown to generate changes in LDL particles similar to those occurring in endothelial cells, including lipid peroxidation and extensive hydrolysis of phosphatidylcholine [28]. To evaluate the effects of oxidised LDL on beta cells, LDL particles were oxidised by copper as performed in many reports [6, 29, 30]. Our previous study had shown that culture of beta cells for 72 h with oxidised LDL containing 2 mmol/l cholesterol (physiological concentration), diminished the insulin level and cell survival [13]. Here, reduction in insulin expression and cell integrity was already visible in cells exposed for 72 h to 1 and 1.5 mmol/l cholesterol of oxidised LDL, but the effect was less pronounced than with 2 mmol/l cholesterol of modified LDL (ESM Fig. 1). As previously shown, 0.5 mmol/l oxidised LDL was not efficient in triggering harmful effects on the cells (ESM Fig. 1) [13]. The decrease in insulin expression achieved by oxidised LDL occurred at a time point at which cell death was not yet apparent (ESM Fig. 2) [13]. This implies that impaired levels of the hormone are not merely the consequence of a reduced number of cells caused by apoptosis. While native LDL can exert deleterious effects on beta cell secretion and proliferation above 3 mmol/l (A. Abderrahmani and D. Favre, unpublished observations) [16], the unmodified lipoproteins at 2 mmol/l cholesterol triggered no effect on beta cell function [13, 14]. For these reasons, we chose this cholesterol concentration to carefully investigate the effects of oxidised LDL. We found a two- to threefold increase in levels of ICER isoforms in MIN6 and INS-1 cells, as well as in isolated rat islets cultured with oxidised LDL

(Fig. 1a). Two ICER isoforms (ICER and ICER Iγ) that result from alternative splicing of *CREM* mRNA have been described [31]. These two isoforms act as transcriptional repressors whose abundance varies within cell types and species [31]. MIN6 cells produce one ICER isoform, while INS-1E cells contain the two forms of the repressor (Fig. 1b). An increase in the contents of all ICER isoforms by oxidised LDL was confirmed at the protein level by western blotting in the two cell types (Fig. 1b). As anticipated (Fig. 1a, b), neither the protein nor mRNA levels of the repressor were augmented in response to native LDL. ICER is a transcriptional passive repressor, which inhibits the expression of genes that contain a CRE in their promoter region [24, 32]. This is achieved by competing with CREB transcriptional activators for binding to the CRE sequence [24, 32]. To assess the activity of ICER in response to oxidised LDL, MIN6 cells were transfected with the luciferase reporter construct driven by two CRE sequences linked upstream to a SV40 promoter (CREluc). While native LDL did not modify luciferase activity, oxidised LDL treatment strikingly decreased CREluc activity, suggesting that repressor activity of ICER was elevated (ESM Fig. 3). The expression of target genes of ICER was next quantified to confirm the increased ICER activity in response to oxidised LDL. The insulin gene [27], *IB1* (also known as *MAPK8IP1*) [23] and four genes of the secretory machinery, the GTPases *Rab3a* and *Rab27a* and their effectors *Slp4* and *Noc2* [25], are known targets of ICER. In line with the increase in ICER abundance, expression of all these target genes was diminished in cells cultured with oxidised LDL, but not in those cultured with native particles (Fig. 2a–f). To clearly establish whether ICER is responsible for these effects, we blocked expression of the repressor using siRNAs (si-*Icer*). The efficacy and specificity of the si-*Icer* in beta cells has already been tested in a former study [25]. The mRNA of *Ib1* and insulin in cells cultured with oxidised LDL was partially restored upon silencing of *Icer* (Fig. 2a, b). In contrast, silencing of *Icer* was able to fully restore the levels of the four genes of the secretory machinery (Fig. 2c–f).

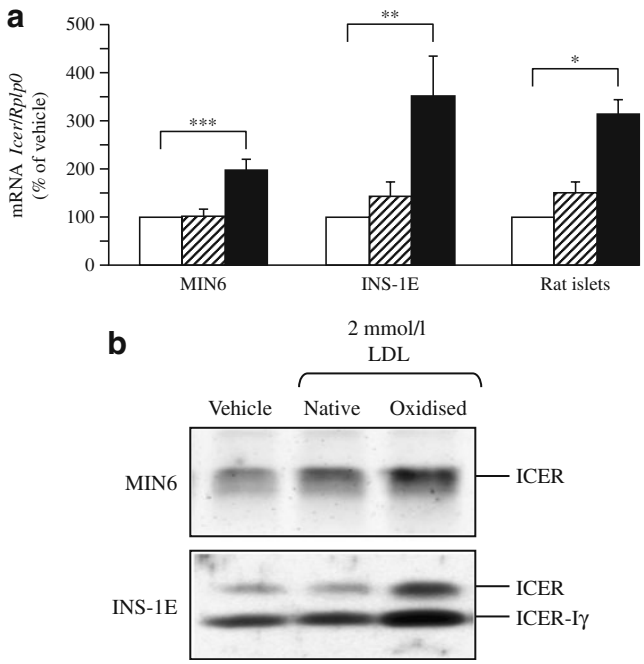

Fig. 1 mRNA and protein levels of ICER in response to oxidised LDL. **a** The mRNA level of *Icer* was quantified by quantitative real-time PCR using total RNA from MIN6, INS-1E and isolated rat islet cells cultured for 72 h with vehicle (white bars), native LDL (hatched bars) or oxidised LDL (black bars). mRNA was normalised against the housekeeping gene, *Rplp0*, and expression from cells cultured with vehicle was set to 100%. Data are mean±SEM of three independent experiments; *$p<0.05$, **$p<0.01$ and ***$p<0.001$. **b** Quantification of ICER content in response to oxidised LDL. Total protein from cells that were cultured with native or oxidised LDL 2 mmol/l cholesterol was subjected to western blotting analysis. Blot shows results of a representative experiment (one of three)

We next investigated the causal role of ICER in beta cell dysfunction and death triggered by oxidised LDL. The decreased expression of *Ib1* caused by oxidised LDL is responsible for sustained induction of the JNK pathway [13]. Activation of this signalling cascade by oxidised LDL leads in isolated rat islets and MIN6 cells to apoptosis through a mechanism involving reduced expression of B-cell CLL/lymphoma 2 [13]. Consistent with the restored mRNA *Ib1* expression, blockade of ICER protected the cells against apoptosis evoked by oxidised LDL (Fig. 3a). As previously reported [14], we found that oxidised LDL compromise glucose-induced insulin secretion (ESM Fig. 4). *Rab3a*, *Rab27a*, *Slp4* and *Noc2* play major role in the control of insulin exocytosis [25]. This led us to assume that the late steps of insulin exocytosis were impaired in the presence of modified LDL. Exogenously produced hGH has previously been shown to be targeted to secretory granules and to be co-released with insulin after triggering of exocytosis [33]. As, in contrast to insulin, hGH biosynthesis is not affected by oxidised LDL, secretion of this hormone was used to directly monitor the exocytotic process in cells cultured with the lipoproteins. In addition, the use of hGH as a reporter for exocytosis allows selective assessment of the secretory process in the fraction of cells that are co-transfected with the siRNAs. Potassium chloride (KCl) is a potent insulin secretagogue that triggers exocytosis of insulin-containing vesicles through direct depolarisation of the beta cell membrane [34]. Culture of the cells with oxidised LDL altered secretion elicited by KCl (Fig. 3b). This result suggests a defect in the distal steps of the secretory process, which could be the consequence of reduced expression of *Rab3a*, *Rab27a*,

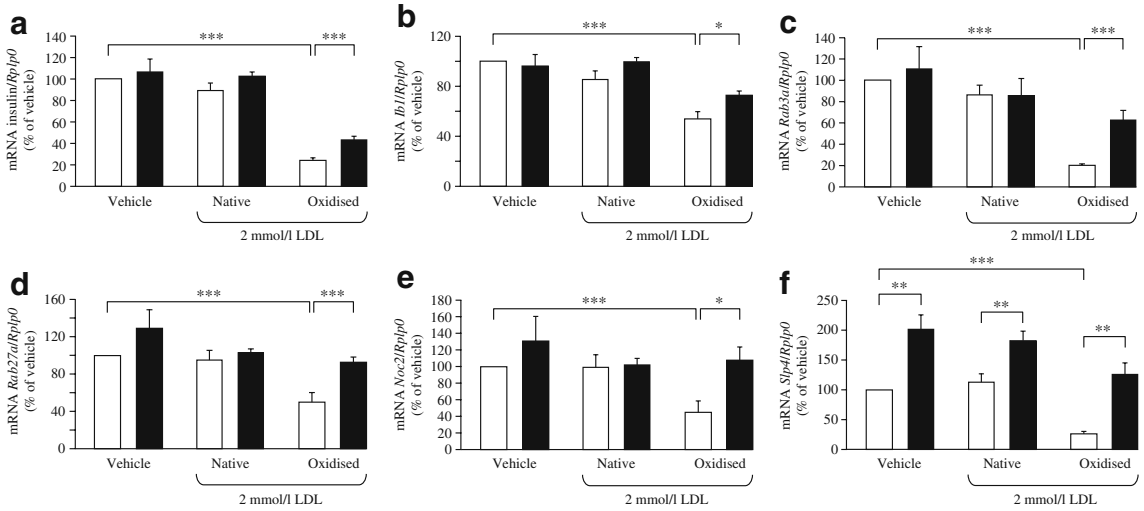

Fig. 2 Expression of ICER target genes in the presence of oxidised LDL. mRNA of (**a**) insulin, (**b**) *Ib1*, (**c**) *Rab3a*, (**d**) *Rab27a*, (**e**) *Noc2* and (**f**) *Slp4* was quantified in MIN6 cells exposed for 72 h to human native LDL or oxidised LDL containing 2 mmol/l cholesterol. The cells were transfected with duplexes of control siRNA (si-*Gfp*) (white bars) or siRNA directed specifically against ICER (si-*Icer*, black bars). mRNA levels were normalised against *Rplp0* and expression from cells cultured with vehicle were set to 100%. Data are the mean±SEM of three independent experiments; *$p<0.05$, **$p<0.01$ and ***$p<0.001$

Slp4 and *Noc2*. Secretion elicited by KCl was completely restored when expression of *Icer* was suppressed (Fig. 3b), confirming the direct role of the repressor in perturbation of the late steps of insulin secretion caused by oxidised LDL.

Antioxidant treatment prevents induction of Icer and oxidised LDL-mediated beta cell failure Oxidised LDL are potent inducers of oxidative stress in several cell types [17, 18]. Oxidative stress is a metabolic process that consists of formation of reactive oxygen species (ROS). In physiological concentrations, endogenous ROS help to maintain homeostasis [35]. However, when ROS accumulate in excess for prolonged periods as observed for all the environmental stressors related to diabetes, they trigger adverse effects [18, 36–39]. Incubation of MIN6 cells with oxidised LDL elicited a twofold increase in superoxide production (Fig. 4a). Similar levels were reached in cells exposed to hydrogen peroxide for 1 h, indicating that oxidised LDL are potent inducers of oxidative stress in insulin-secreting cells (Fig. 4a). We then investigated whether oxidative stress can induce expression of *Icer*. Treatment with hydrogen peroxide resulted in four-, two- and 1.7-fold induction of the expression of *Icer*, in MIN6 cells, and isolated islets of rat and humans, respectively (Fig. 4b). To confirm the role of oxidative stress in induction of *Icer* achieved by oxidised LDL, the cells were co-cultured with the modified lipoproteins and the antioxidant, *N*-acetylcysteine (NAC) at 1 mmol/l. NAC is the acetylated precursor of the amino acid L-cysteine and reduced glutathione. NAC exerts a powerful antioxidant action by elevating the level of glutathione. Addition of NAC abolished induction of *Icer* caused by oxidised LDL in MIN6, and in isolated islets of rat and humans (Fig. 4c-e). Co-treatment with NAC enabled partial protection of the cells against loss of insulin level (Fig. 5a) and completely restored glucose-induced insulin secretion executed by oxidised LDL (Fig. 5b). Restoration of the secretory capacity was in line with the rise in expression of the genes of the secretory machinery (ESM Fig. 5). Co-incubation of the cells with NAC prevented cell death triggered by oxidised LDL in MIN6 and isolated rat islets (Fig. 5c, d). HDL-cholesterol particles antagonise the adverse effects of oxidised LDL in beta cells [13, 15] and display antioxidant properties [40]. Co-culture of MIN6 cells in the presence of HDL prevented the induction of *Icer* caused by oxidised LDL (Fig. 6), pointing to the repressor as an important link coupling oxidative stress to beta cell failure achieved by the modified LDL.

Discussion

Evidence of adverse effects of pro-atherogenic oxidised LDL on beta cells [13, 15, 16] is now growing. Several independent in vitro studies agree in pointing to a deleterious impact of these modified lipoproteins on insulin level, glucose-induced insulin secretion and ultimately cell survival in different insulin-secreting and islet cells [13, 15, 16]. Such harmful effects, which are potentiated by inadequate levels of and/or inactive HDL,

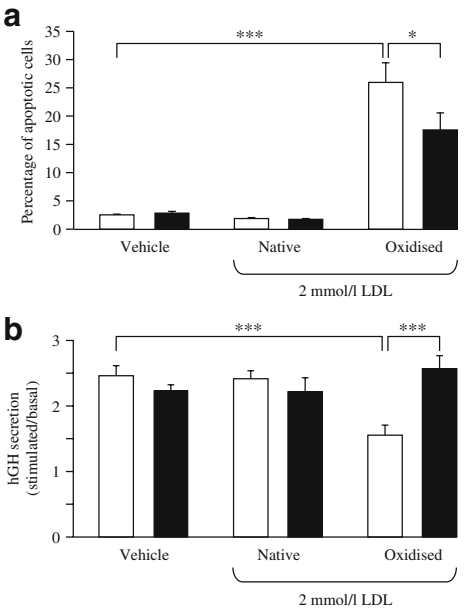

Fig. 3 Effect of *Icer* silencing on secretion and survival. **a** MIN6 cells were transfected with a control RNA duplex (si-*Gfp*) (white bars) or with si-*Icer* (black bars). Cells were then cultured for 72 h with native or oxidised LDL 2 mmol/l cholesterol. The fraction of cells undergoing apoptosis was determined by scoring the percentage of cells with picnotic nuclei. **b** Measurement of insulin exocytosis. MIN6 cells were transiently transfected with hGH together with the control siRNA (white bars) or si-*Icer* (black bars), and cultured as above (**a**). Secretion of hGH was elicited by pre-incubating cells for 30 min in KRB containing 2 mmol/l glucose and then incubating them with KCl 30 mmol/l. The amount of hGH released into the medium during the incubation period and remaining in the cells was measured by ELISA. The results are expressed as fold increase of stimulated over basal. Data (**a**, **b**) are the mean±SEM of three independent experiments; $*p<0.05$ and $***p<0.001$

are thought to promote and contribute to the progression of diabetes [13, 15]. Our aim here was to understand the mechanism through which oxidised LDL leads to beta cell dysfunction. ICER is a basic leucine zipper transcriptional factor that negatively regulates expression of numerous genes containing a CRE sequence within their promoter. The lack of an activation domain makes ICER into a passive transcriptional repressor [24]. The inhibitory role of ICER is achieved at certain levels of abundance. Thereafter, the repressor acts by competing with transcriptional activators and preventing their binding to the CRE [24]. Induction of ICER is a physiological process that permits levels of some hormones in endocrine or neuroendocrine cells to return to basal [41]. However, persistent expression of this repressor can occur in response to a deleterious milieu [41]. In this study, we found that abnormal induction of ICER links oxidised LDL to beta cell dysfunction. Selective reduction of ICER contents partially prevented the loss of insulin expression and cell death evoked by oxidised LDL. Loss of specialised beta cell functions caused by the modified lipoproteins preceded cell death. While ICER is responsible for beta cell failure and death, our observations imply that beta cell dysfunction driven by the repressor is established prior to cell death. Variation in islet brain 1 levels is critical for maintaining appropriate insulin content and cell survival [13, 22]. As observed in our former study [13], the islet brain 1 level was dramatically diminished in the face of oxidised LDL, confirming that loss of islet brain 1 function mediates at least some of the damages to beta cells provoked by the modified lipoprotein. Reduction of islet brain 1 abundance inhibits the insulin level and increases apoptosis by permitting activation of the JNK pathway [22]. While ICER can directly target the gene coding for insulin [42], it could also cause the loss of insulin expression and cell death indirectly by reducing islet brain 1 content and stimulating the JNK pathway. However, restoration of levels of the scaffold protein was incomplete upon silencing of *Icer*. This coincided with a partial recovery in insulin production and survival. This result could not be attributed to efficacy of silencing, because si-*Icer* was able to fully restore the mRNA level of the two other ICER target genes, *Noc2* and *Rab27a*. These observations suggest that additional factors that are independent of ICER could have contributed to the decreased abundance of islet brain 1 provoked by the modified particle. However, as observed for chronic hyperglycaemia [25], it is mostly induction of ICER that operates dysregulation of genes controlling insulin secretion, and thereby the defective secretory capacity of beta cells caused by oxidised LDL. We found that silencing of *Icer* permitted full protection against impairment of stimulated secretory capacity triggered by oxidised LDL, a finding that correlates with fully restored expression of *Noc2* and *Rab27a*.

The major role of ICER in mediating the adverse effects of oxidised LDL led us to seek the mechanism responsible for its induction. Many in vitro studies show that oxidised LDL favour production of ROS and thus oxidative stress in various cell types [17, 18]. Furthermore, elevated levels of oxidised LDL correlate with an increase in certain markers of oxidative stress, such as heat shock protein 70 in patients with newly diagnosed and long-standing diabetes [43]. For these reasons, we investigated the role of oxidative stress in stimulating production of ICER. In this regard, we first found that oxidised LDL evoked production of superoxide in beta cells. Second, culture of cells with hydrogen peroxide elicited an increase in ICER levels. Finally, NAC antioxidant treatment abrogated the induction of ICER caused by oxidised LDL. Consistent with this result, mRNA expression of ICER target genes *Rab3a*, *Rab27a*, *Slp4* and *Noc2* was restored. This was further accompanied by full recovery of glucose-induced insulin secretion.

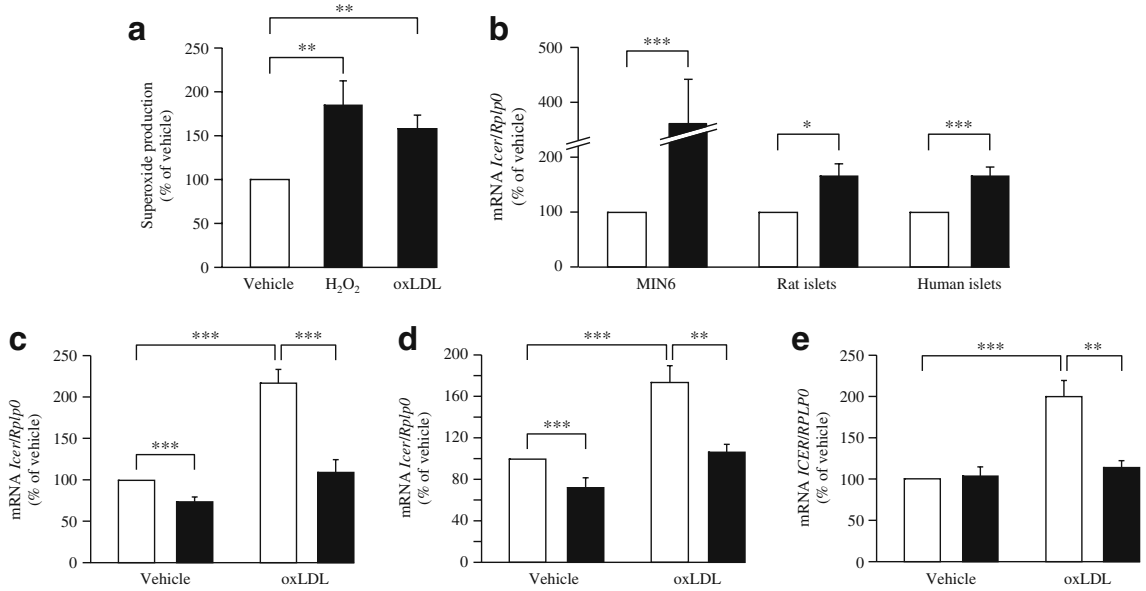

Fig. 4 Role of oxidative stress in the induction of *Icer* by oxidised LDL. **a** Production of superoxide in response to oxidised LDL. Extracellular ROS were measured by luminescence. MIN6 cells were cultured for 1 h with oxidised LDL 2 mmol/l (oxLDL) or hydrogen peroxide 150 μmol/l (H_2O_2). Superoxide production in cells cultured with vehicle condition was set to 100%. **b** Effects of hydrogen peroxide on the expression of *Icer*. The mRNA level of *Icer* was quantified by quantitative real-time PCR from MIN6 cells, isolated rat islets cultured for 4 h and human islets cultured for 0.5 h with hydrogen peroxide 150 μmol/l (black bars). mRNA levels were normalised against the *Rplp0* and expression from cells cultured with vehicle was set to 100%. **c–e** Effects of the antioxidant NAC on expression of *Icer* evoked by oxidised LDL. The mRNA level of *Icer* was quantified by quantitative real-time PCR using total RNA that was prepared from (**c**) MIN6 cells, and isolated rat (**d**) and human (**e**) islets cultured for 4 h with oxidised LDL 2 mmol/l cholesterol. The cells were co-incubated with vehicle (white bars) or NAC (black bars) 1 mmol/l for MIN6 and isolated rat islets (**c, d**) or 10 mmol/l for human islets (**e**). Results were normalised against *RPLP0* and expression from cells cultured with vehicle was set to 100%. **a–e** Data are the mean±SEM of three independent experiments; *$p<0.05$, **$p<0.01$ and ***$p<0.001$

However, NAC treatment was partially effective in preventing the loss of insulin transcript accomplished by oxidised LDL. This observation leads us to assume that another mechanism, parallel to oxidative stress, accounts for the partial reduction of insulin caused by the modified LDL. Administration of NAC strongly improved survival of MIN6 cells in the presence of oxidised LDL, supporting the notion of a cytoprotective role of the antioxidant

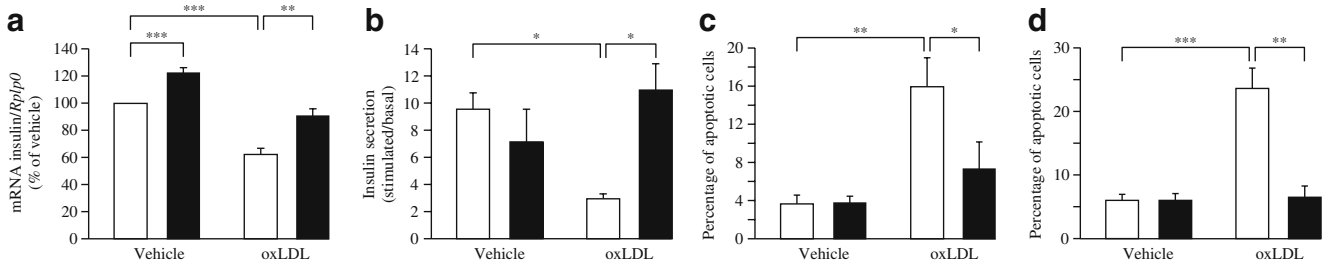

Fig. 5 Counteractive effect of NAC on adverse effects of oxidised LDL on beta cell functions. **a** Insulin expression in the presence of NAC. MIN6 cells were cultured for 72 h with oxidised LDL (oxLDL) 2 mmol/l cholesterol in the presence of vehicle (white bars) or NAC 1 mmol/l (black bars). The insulin mRNA level was determined by quantitative real-time PCR. mRNA levels were normalised against *Rplp0* and the results obtained in cells cultured with vehicle were set to 100%. **b** Assessment of glucose-induced insulin secretion. MIN6 cells were cultured for 72 h with oxidised LDL 2 mmol/l cholesterol plus vehicle (white bars) or NAC 1 mmol/l (black bars). For measurement of glucose-induced insulin secretion, the cells were pre-incubated for 60 min in KRB containing 2 mmol/l glucose (basal) and successively incubated in the same buffer or in KRB containing 20 mmol/l glucose (stimulated). The amount of insulin release and cellular contents during the incubation period were measured by EIA. Results are expressed as the ratio between the amount of insulin released into the medium under stimulatory and basal conditions over basal condition. **c**, **d** Cell survival in the presence of NAC. MIN6 cells (**c**) and dispersed rat islet cells (**d**) were cultured for 72 h with oxidised LDL 2 mmol/l cholesterol plus vehicle (white bars) or NAC 1 mmol/l (black bars). The fraction of cells undergoing apoptosis was determined by scoring cells with pycnotic nuclei. **a–d** Data are expressed as the mean±SEM of three independent experiments; *$p<0.05$, **$p<0.01$ and ***$p<0.001$

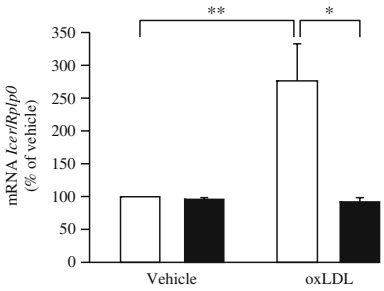

Fig. 6 Effects of HDL on oxidised LDL-induced *Icer* expression. MIN6 cells were cultured for 4 h with oxidised LDL (oxLDL) 2 mmol/l cholesterol in the presence of vehicle (white bars) or 1 mmol/l HDL-cholesterol (black bars). *Icer* mRNA was determined by quantitative real-time PCR. mRNA levels were normalised against *Rplp0* and the results obtained in cells cultured with vehicle were set to 100%. Data are the mean±SEM of three independent experiments; *$p<0.05$ and **$p<0.01$

against the deleterious effects of the modified lipoprotein in beta cells. Even though primary beta cells were not examined, the protective effect of NAC was almost complete in isolated islets. Activation of the JNK pathway and decrease of *Bcl2* expression are partially responsible for apoptosis elicited by oxidised LDL [13]. Generation of oxidative stress could therefore be the leading upstream cause of activation of JNK pathway and decrease of B-cell CLL/lymphoma 2 levels, in a mechanism that could also involve ICER.

HDL particles are potent antioxidants [40] and exert many beneficial effects in beta cells. They not only stimulate beta cell function [15], but also counter loss of beta cell functions and cell survival achieved by oxidised LDL or chronic hyperglycaemia [13, 15, 16]. In line with this cytoprotective role, we found that HDL abolished induction of ICER evoked by oxidised LDL. As observed for oxidised LDL, chronic excess of glucose elicits beta cell dysfunction and death by generating intracellular oxidative stress and elevating ICER levels [25, 44]. Thus, it is very likely that HDL particles counteract adverse effects provoked by prolonged exposure to glucose by inhibiting oxidative stress and consequently blocking ICER production. However, the plasma concentration of HDL particles and their activity are reduced in patients with diabetes or metabolic syndrome [7, 8]. For this reason, much is being done to find out effective therapeutic strategies to elevate plasma HDL. It has, however, been suggested that loss of HDL activity in patients with type 2 diabetes might be the consequence of an oxidised LDL-mediated decrease in lecithin-cholesterol acyltransferase activity [45]. Therefore it is possible that oxidised LDL elicit oxidative stress and, consequently, beta cell failure by directly abolishing the protective effects of HDL. In this case, as long as oxidised LDL are present, improvement of HDL levels in patients may not effectively counteract beta cell failure. If this hypothesis is correct, then the use of other alternative antioxidant approaches to counteract oxidative stress in beta cells may be required. Numerous in vitro and in vivo studies have shown the efficacy of certain antioxidants in preventing beta cell failure. For example, administration of the antioxidant NAC alone or in combination with some vitamins ameliorated glucose-induced insulin secretion in Zucker diabetic fatty rats and *db/db* mice [44, 46]. In contrast, large clinical trials failed to detect an improvement in insulin secretion in patients with type 2 diabetes in response to antioxidant vitamins [47, 48]. However some findings in favour of preventive antioxidant therapy to combat the decline of beta cell function in diabetes have been reported, in particular, a clinical study originally devised with the primary endpoint efficacy of the antioxidant succinobucol in cardiovascular outcomes in patients with acute coronary disease [19]. Interestingly, daily administration of the antioxidant succinobuccol has been found to reduce occurrence of new-onset diabetes by almost threefold [19]. Succinobuccol is derived from probucol, a bisphenol molecule that preserves beta cells by attenuating oxidative stress [49]. NAC is a potent antioxidant, which is widely used as an antidote for hepatotoxicity caused by acetaminophen overdose. At present, a growing body of data supports many other clinical benefits from use of this antioxidant. These include: prevention of chronic obstructive pulmonary disease exacerbation, attenuation of illness from the influenza virus, treatment of pulmonary fibrosis and treatment of infertility in patients with clomifene-resistant polycystic ovary syndrome [50]. Therefore, antioxidant supplementation in a subset of patients with low HDL levels and elevated oxidised LDL could help to prevent beta cell failure and thereby delay the development of diabetes.

Induction of ICER by oxidative stress may represent the unifying mechanism for beta cell failure provoked by chronic hyperglycaemia, hyperlipidaemia and oxidised LDL. For the future design of effective and potent antioxidant preventive treatment, careful examination of their ability to counteract adverse effects of diabetes stressors in beta cells will be required.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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C Loss of ICER accounts for the sustained adipose CREB activity in obesity

C Loss of ICER accounts for the sustained adipose CREB activity in obesity

C.1 Introduction

Insulin resistance is the initial measurable defect in the pathogenesis of T2D and is correlated with the extent of visceral adiposity in obese patients (83). The normal function of adipose tissue is perturbed with excessive weight and there is accumulating evidence suggesting that adipocyte dysfunction plays a major role in the development and progression of insulin resistance (84). Increased visceral adiposity is associated with impaired glucose uptake by the adipocytes, owing in part to the reduced expression of GLUT4 (30;85). In addition to the altered glucose disposal, adipocytes produce and secrete lower amounts of the insulin-sensitizer adiponectin. The subsequent decline in circulating levels of adiponectin is thought to participate to the progressive loss of insulin sensitivity in obese individuals (37;86). Interestingly, an important mechanism responsible for the impaired expression of GLUT4 and adiponectin in obesity has been revealed and involves the CREB proteins (76). The transcriptional activity of the CREB factors is abnormally augmented in the adipocytes of obese mice, ensuing in the expression of the transcription factor ATF3. Induction of this transcriptional repressor, in turn suppresses the levels of its target genes GLUT4 and adiponectin. Accordingly, mice overexpressing a dominant negative form of CREB specifically in the adipocytes exhibit an improved insulin sensitivity and glucose homeostasis under condition of high fat diet (76).

One of the major controllers of the activity of the CREB factors is the transcription factor ICER. This transcriptional repressor represses the expression of genes by competing with the activating factors of the CREB family for the binding of CRE sequences (61). All these observations led us to question whether the stimulated expression of ATF3 by the CREB factors and the subsequent

loss of adiponectin and GLUT4 levels could be the consequence of a decline in ICER in the adipose tissue of obese individuals.

C.2 results

In order to study the potential role of ICER in the adipocyte dysfunction in obesity, we quantified the expression of this transcriptional repressor, ATF3, GLUT4 and adiponectin in the white adipose tissue (WAT) of mice fed a HFD and in the visceral adipose tissue (VAT) of obese human subjects. The transcript levels of ATF3 were massively augmented in the adipose tissue of the two obese models, resulting in the loss of GLUT4 and adiponectin expression. This activation of ATF3 was accompanied by a marked reduction in the mRNA levels of ICER in the obese adipose tissues (Fig 1). Importantly, these modifications were observed in the adipocytes fraction of the WAT of obese mice, but absent from the stromal vascular fraction which contains mainly pre-adipocytes and macrophages (Fig 2a and b). This observation testifies therefore for the adipocyte specificity of the loss of ICER in obesity. The disappearance of ICER was associated with an elevation in the activity of the CREB factors, as shown by the augmentation in CREB binding in electromobility shift assays (Fig 1c-e).

To demonstrate the ability of ICER to control ATF3 expression in adipocytes, we overexpressed the transcriptional repressor in the adipocyte model 3T3-L1 cells by electroporation techniques. Exogenous expression of ICER markedly decreased ATF3 mRNA levels in adipocyte and non-differentiated 3T3-L1 cells (Fig 3a and b). Finally, the knocking out of ICER transcript by RNA interference favored an augmentation in the mRNA and protein contents of ATF3 (Fig 4a and b).

In conclusion, all these data indicate that the loss of the transcriptional repressor ICER in the obese adipose tissue is responsible for the activation of the transcription factor ATF3 by CREB and the subsequent impairment in GLUT4 and adiponectin expression.

C.3 Contribution

In this paper, the student performed the majority of the experiments. He was helped in his task by his colleagues. The student carried out the preparation of the figures, the statistical analysis of the data and contributed to the redaction of the manuscript. Finally, he designed and performed the major part of the experiments for the correction of the manuscript.

Impaired expression of the inducible cAMP early repressor accounts for sustained adipose CREB activity in obesity

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Impaired expression of the inducible cAMP early repressor accounts for sustained adipose CREB activity in obesity

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Running title: Dysregulation of ICER in obesity.

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Abstract

Objective Increase in adipose cAMP response binding proteins (CREB) activity promotes adipocyte dysfunction and thereby, systemic insulin resistance in obese mice. This is achieved by increasing the expression of activating transcription factor 3 (ATF3). In this study we investigated whether impaired expression of the inducible cAMP early repressor (ICER), a transcriptional antagonist of CREB, is responsible for the increased CREB activity in adipocytes of obese mice and humans.

Research Design and Methods Total RNA and nuclear proteins were prepared from visceral adipose tissues (VAT) of human lean or obese subjects, and white adipose tissues (WAT) of C57Bl6-Rj mice that were fed with normal or high fat diet for 12 weeks. The expression of genes was monitored by real-time PCR, western blotting and electromobility shift assays. RNA interference was used to silence the expression of Icer.

Results The expression of ICER was reduced in VAT and WAT of obese humans and mice, respectively. Diminution of Icer was restricted to adipocytes and was accompanied by a rise in Creb activity and elevation of *Atf3*, leading to repression of the Atf3 target genes *Adipoq* and *Glut4*. Overexpression of Icer in 3T3-L1 adipocytes as well as fibroblasts decreased the Atf3 content while silencing of Icer increased the expression of Atf3, indicating a mechanistic link between the level of the two transcription factors.

Conclusion Impaired expression of Icer contributes to elevation in Creb target genes and therefore contributes to the development of insulin resistance in obesity.

Accumulation of visceral fat in obesity is a strong risk factor for life-threatening diseases such as type 2 diabetes, atherosclerosis, polycystic ovary syndrome, non-alcoholic fatty liver disease and certain types of cancer (1-4). Increased adiposity is associated with impairment in glucose uptake in adipocytes, which in part result from the diminished expression of GLUT4 (5). Parallel to defective glucose metabolism, adipocytes display reduced production of the insulin-sensitizer adiponectin (Adipoq) (6;7). This dysregulation is considered as part of a crosstalk linking adiposity to overall insulin resistance in obesity (8). An important mechanism responsible for decreased level of Glut4 and Adipoq in obesity is now advanced (9). This involves the cAMP response element binding proteins (CREB) transcriptional factors (9). Transcriptional activity of Creb is abnormally elevated in adipocytes of obese mice fed with a high fat diet (HFD) resulting in elevation of the expression of its target gene the activating transcription factors 3 (Atf3) (9). In turn, Atf3 suppresses the expression of Glut4 and Adipoq and thereby, disrupts insulin action in adipocytes and causes systemic insulin resistance (9). Obese mice that express a dominant-negative form of Creb in adipocytes have improved whole body-insulin sensitivity and glucose homeostasis (9). Furthermore, the transgenic mice are protected against hepatic steatosis and adipocytes inflammation (9).

The activity of the CREB factors is finely tuned by the level of the inducible cAMP early repressor (ICER). ICER is a member of the cAMP-responsive element modulator family (CREM). ICER represses genes transcription by binding to the CRE sequence within their promoters (10). Typically, ICER plays as a dominant-negative role by competing with all cAMP responsive transcriptional activators of the CREB, CREM and activating transcription factor families for binding to CRE (11). Induction of ICER is

part of an adaptive mechanism that allows production of hormones to return to basal state (11). Elevation in the ICER level is normally transient and dysregulation in the expression of this repressor leads to diverse pathologies (11-14). In this report, we investigated whether a diminished Icer level could be responsible for the increase of ATF3 and, consequently, the loss of Adipoq and Glut4 expression in the adipose tissue of obese individuals.

Research Design and Methods

Additional information concerning the hyperinsulemic-euglycemic clamps, suppliers of chemicals and antibodies as well as for the methods of cell culture, transfection, western blotting and electromobility shift assays (EMSA) can be found in the online appendix.

Animal experimentation- All procedures on mice were performed according to the Swiss legislation for animal experimentation. Six-week-old C57Bl6-Rj male mice were obtained from the Janvier Breeding Centre (Le Genest-Saint-Isle, France). They were placed for 12 weeks randomly upon either a standard diet including proteins 28%, carbohydrates 60% and fat 12% (SD, Provimi Kliba, Penthalaz, Switzerland) or a fat-enriched diet with the following composition proteins 21%, carbohydrates 24%, fat 55% (Harlan Teckland, Oxon, UK).

Biopsies and RNA preparation- Approximately 5 cm^3 of VAT was obtained at the level of the omentum from five obese white women (BMI >35 kg/m^2) who were referred consecutively over a period of 24 months for weight reduction surgery and lean individuals (24< BMI <28 kg/m^2). The criteria for exclusion and phenotyping are those previously described (15). Tissue samples and total RNA were prepared exactly as previously described (16). Total RNA was isolated from epididymal visceral adipose tissue of mice (immediately frozen in liquid N_2 and stored at -80°C after dissection) with TriPure isolation reagent (Roche).

Real-time quantitative PCR- Preparation of total RNA and real-time PCR were conducted exactly as described (12). Primer sets are available upon request.

Statistical analysis- The experiments including more than two groups were analyzed by ANOVA or with the non parametric equivalent Kruskal-Wallis test.

Results

Samples of white adipose tissues (WAT) were prepared from obese mice fed with a high fat diet (HFD) for 16 weeks. This mice model of obesity mimics metabolic disorders observed in human (17;18). As the result of the diet, mice indeed gained significantly more body weight (n= 8, 23.1±6.1 vs. 32.1±1 grams, $p<0.05$), were hyper-insulinemic (n=8, 15±7 vs. 53±9 μU/ml, $p<0.05$) and hyperglycaemic (n= 8, 134±13.5 vs. 168±17 mg/dl, $p<0.05$) compared to littermates fed with chow diet. The HFD mice were insulin resistant as indicated by a marked decrease in glucose infusion rate (GIR, n=8, 94±19 vs. 33±5 mg/kg/min, $p<0.05$). In this model, elevation in the transcriptional activity of Creb has been shown to promote insulin resistance in adipose tissue (9). The defect in insulin sensitivity is caused by the increase in the expression of Atf3, which in turn, downregulates the expression of Glut4 (9). In agreement with these data, we found an increase in the *Atf3* mRNA level whereas the expression of *Glut4* decreases in WAT of obese mice when compared to control animals (Fig. 1a). Elevation of *Atf3* also partially accounts for systemic insulin resistance (9). This is achieved by a subsequent diminution in the mRNA level of its target gene *Adipoq* (9). In line with this finding, we confirmed reduction in the *Adipoq* level in WAT of obese mice (Fig.1a). Increase in the expression of the Creb target gene Atf3 in WAT was associated with a striking diminution in the *Icer* mRNA level (Fig. 1a), thus supporting a role for Icer in the dysregulation of adipose tissues. The drop in Icer level and the subsequent changes in the expression of *Atf3*, *Glut4* and *Adipoq* were observed in WAT of obese animals under fasting condition (Fig. 1a). Similar results were found using WAT from animals under fed conditions (data not shown), indicating that impaired Icer level in HFD-induced

obese mice is independent from the nutritional status. Samples of adipose tissues from human lean and obese subjects were prepared to confirm the data obtained in mice. Since it is the excess of visceral fat rather than subcutaneous, which is associated with an increased risk of developing cardiovascular disease and metabolic syndrome, we chose to quantify the expression of genes in visceral adipose tissue (VAT) (19). As observed in mice, the *ICER* mRNA level was decreased in VAT of obese individuals (Fig. 1b). This was associated with an increase in the expression of the CREB target gene *ATF3* and decreased levels of *ADIPOQ* and *GLUT4* (Fig. 1b). EMSA were next done to confirm changes in binding activities of Icer and Creb in adipose tissues of obese mice. Nuclear proteins from WAT of obese mice were incubated with a labelled oligonucleotide corresponding to the cAMP response element (CRE) (12). A fast migrating band was observed in WAT of control mice, which was markedly reduced in obese mice (Fig. 1c). On the other hand, a slower migrating pattern appeared in WAT of obese mice that is almost undetectable in control mice (Fig. 1c). The binding activities were specific to CRE as a 100 fold excess of the unlabeled probe, but not of unspecific binding sequences, efficiently prevented the formation of the two bands (Fig. 1d and 1e). The lower band was identified as Icer because it co-migrates with exogenously expressed ICER-Iγ and the formation of this complex was selectively abrogated by the addition of an anti-CREM antibody (Fig. 1d). In the presence of the anti-Creb1 antibody, the migration of the upper band observed in WAT of obese mice was slower (Fig. 1e). This result indicates that nuclear extracts of WAT from obese mice display an increase in Creb binding activity with a concomitant reduction in the expression of Icer. Creb activity, and consequently the expression of its target genes are required for adipogenic program (20;21). Various

stimuli including insulin can elicit Creb activity (20;21). In line with this we found that insulin infusion elicited a 16 fold increase in the expression of Icer in control mice (supplementary Fig. 1). As anticipated insulin-induced Icer expression was abolished in insulin resistant WAT of obese mice, indicating that induction of Icer is also defective in obese animals.

The drop of Icer was only observed in the adipocyte fraction of obese mice, whereas the expression of the repressor in the stroma vascular fraction was similar between obese and control mice (Fig. 2a). The reduced Icer content in adipocytes paralleled the rise in the mRNA level of *Atf3* (Fig. 2b) and the diminution in Adipoq and Glut4 levels (Fig. 2b).

We then attempted to confirm the negative regulation of Atf3 by Icer. To this end, a plasmid encoding for Icer-Iγ was introduced by electroporation into 3T3-L1 adipocytes. Western blotting and EMSA experiments revealed a high expression of Icer (supplementary Fig. 2). Overexpression of Icer-Iγ led to reduction in the mRNA level of *Atf3* (Fig. 3a). IBMX is a phosphodiesterase inhibitor that elicits activation of the cAMP/PKA pathway and thereby the activation of CREB. As anticipated, culture of 3T3-L1 adipocytes with IBMX for 4 h evoked a rise in the level of *Atf3* (Fig. 3a). Overexpression of Icer-Iγ alleviated IBMX-induced expression of *Atf3* (Fig. 3a). Similar results were obtained in 3T3-L1 fibroblasts (Fig. 3b), indicating that regulation of *Atf3* by Icer is conserved in most cell types. On the other hand, we found that silencing of Icer by RNA interference elevates the level of Atf3 (Fig. 4a and 4b) confirming the negative regulation of Icer on *Atf3* expression.

Discussion

Increase in the Atf3 level inhibits the expression of Adipoq and Glut4 in adipocytes and thereby promotes systemic insulin resistance of HFD-induced obese mice (9). Our data not only validate this observation but also confirmed dysregulation of these genes in human adipose tissues of obese individuals. Augmentation of Creb activity has been shown to be responsible for the elevation in Atf3 expression (9). The positive regulation of *Atf3* by Creb is direct as the proximal promoter of Atf3 contains a Cre sequence that directly bind CREB (9). Icer contains a DNA binding domain for Cre and, consequently, shares similarities with CREB (10;22;23). However, the properties of Icer diverge from those of the CREB factors because it lacks the activating domain (10;22;23). For this reason, Icer functions as a passive repressor that prevents the transcriptional activators CREB from binding to the Cre sequence (10;22;23). In this report, we provide evidence that dysregulation of Icer in adipocytes is responsible for CREB-induced expression of Atf3 in obesity. First, diminution of Icer correlated with the increase in Atf3 expression in adipocytes from HFD-induced obese mice and human obese individuals. The drop in the Icer level in adipose tissues elevates the Atf3 expression by increasing Creb activity. EMSA experiments indeed showed that reduced Icer expression was accompanied by an increase in Creb binding activity in adipose tissues of obese mice. Second, *in vitro* manipulation of the level of Icer confirmed Atf3 as a target gene. On the one hand, overexpression of Icer in 3T3-L1 adipocytes or fibroblasts inhibited the expression of Atf3. On the other hand, the level of Atf3 was augmented upon silencing of Icer. Based on these findings we propose that reduction in the Icer content in adipocytes could permit CREB to occupy the Cre located in the

promoter of the *Atf3* gene. Induction of the latter gene would in turn silence the expression of Adipoq and Glut4.

Creb activity, and consequently the expression of Icer, can be induced by various stimuli including insulin (20;21). With this respect we found that insulin elicited a rise in the expression of Icer in control animals whereas insulin-induced expression of the repressor was abrogated in insulin resistant WAT of obese mice. The fact that the expression of Icer in WAT of obese animals was reduced both in fed and fasting conditions, supports a defective induction of Icer, and therefore impaired negative control of Creb activity upon stimulation. This dysregulation could be responsible for persistent increased CREB activity, which in turn could lead to the constitutive increase in Atf3 expression and the subsequent decrease in Glut4 and Adipoq levels. Further studies are now needed to identify the mechanism leading to impaired expression of Icer in obesity. We believe that such investigations may pave the way for the design of novel therapeutic strategies or for improvement of existing approaches for recovering normal adipose function and insulin sensitivity.

Acknowledgments

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Figures legends

Fig. 1: Examination of ICER level in adipose tissues of obese mice and human subjects. The mRNA level of ICER was quantified by quantitative real-time PCR in **a**) WAT of control mice fed with a standard diet (C, *white bars*) and HFD-induced obese (H, *filled bars*) and **b**) in VAT of human lean (L, *white bars*) and obese individuals (O, *filled bars*). The expression of the ICER target gene ATF3, ADIPOQ and GLUT4 was also monitored in adipose tissues of obese mice and humans. In all cases, the results were normalised against the housekeeping acidic ribosomal phosphoprotein P0 gene (RPLP0) gene and those of the control mice (C) or lean human (L) cells were set to 100%. Data are the mean ± SEM for 10 mice or 5 humans for each groups that were repeated three times (***, P<0.001; **, P<0.01). **c**) Assessment of the CRE-binding activities. Nuclear extracts of WAT of obese (H) and control mice (C) were incubated with the radioactive probe containing the consensus Cre (CRE). The arrows indicate the major migrating bands. Identification of the CRE-binding activity in the WAT of **d)** control (C) and **e)** obese mice (H). For both **d**) and **e**) panels, the nuclear extracts were omitted in the first lane (-). Where indicated the incubation was performed in the presence of 100-molar excess of unlabeled oligonucleotides corresponding to the consensus CRE sequence (CRE) or of the binding sequences for the Sp1 transcription factor (Sp-1). Before the addition of the labeled oligonucleotide, the nuclear extracts were pre-incubated with antibodies recognizing CREM (CREM) or CREB1 and the unrelated factors Hairy and Enhancer of Split-1 (HES-1), RE-1 silencing transcription factor (REST) and histone deacetylase 1 (HDAC1) as negative controls (Nsp1, Nsp2 and Nsp3, respectively). Nsp:

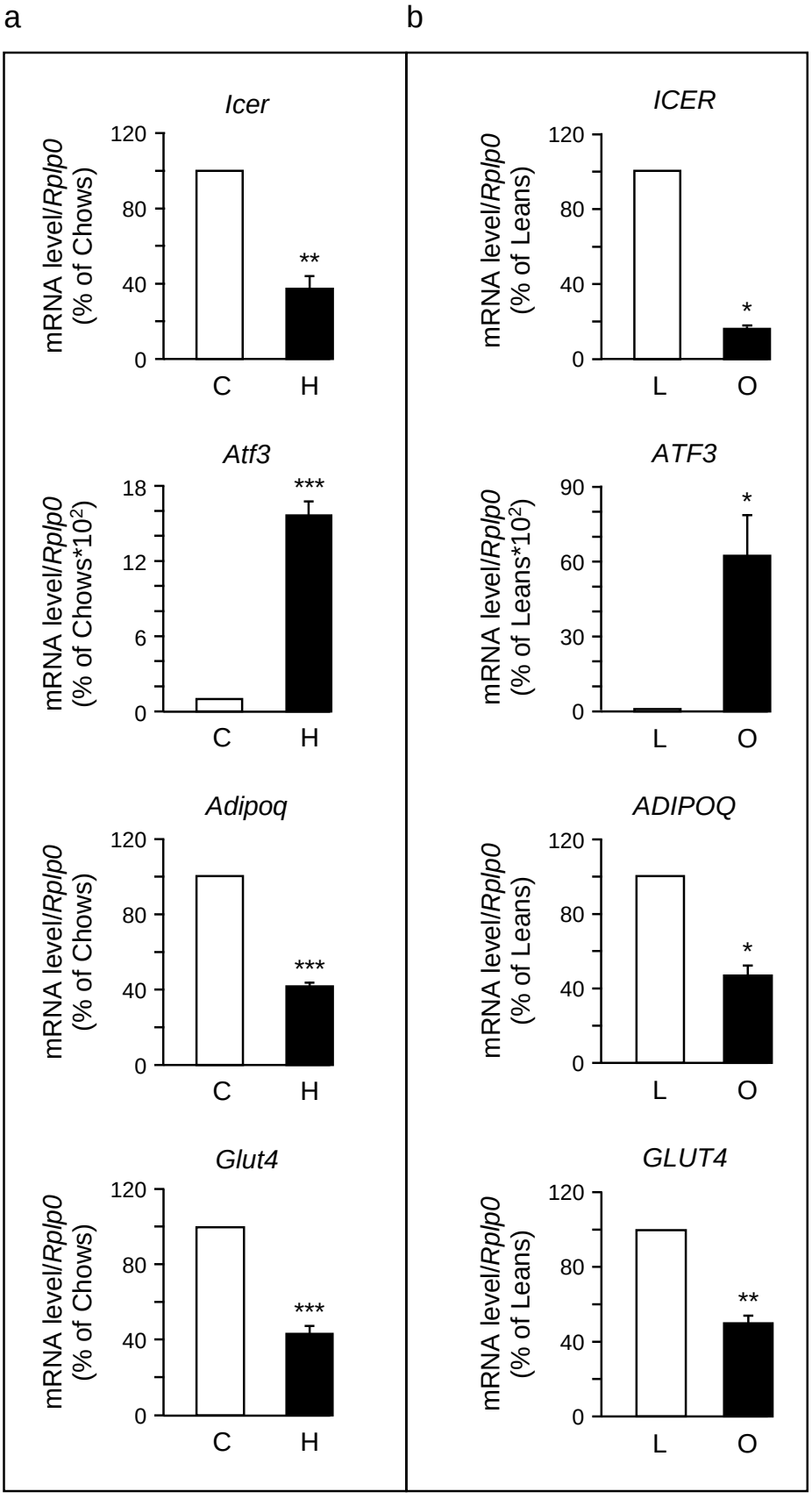
Non specific. For comparison, nuclear extracts of HEK 293T cells transfected with an empty vector (CMV) or with a plasmid producing ICER-Iγ (ICER) were incubated in parallel with the probe. The positions of ICER and of the unbound CRE probe are indicated. The results are representative of three independent experiments.

Fig. 2: Expression of ICER and its target genes in adipocytes. The mRNA level of *Icer* was assessed by quantitative PCR in adipocytes or Stroma vascular fraction (SVF) that were collected from WAT from either control (C) or HFD-induce obese mice (H). **b**) The expression of *Atf3*, *Adipoq* and *Glut4* was measured in adipocytes from control (C) or obese mice (H). The mRNA levels were normalized against the *Rplp0* and the expression levels from controls (C) were set to 100%. Data are the mean of ± SEM of 3 independent experiments (***, P<0.001; **, P<0.01; *, P<0.05).

Fig. 3: Overexpression of Icer reduces the level Atf3. a) The 3T3-L1 adipocytes and **b**) undifferentiated cells were transfected with either a control plasmid (pcDNA3, *white bars*) or with a plasmid coding for Icer-Iγ (ICER, *filled bars*). Total RNAs were prepared from cells that were incubated for 4 h with vehicle (-) or with IBMX to stimulate the expression of *Atf3*. The levels were normalised against the *Rplp0* and the expression levels from cells cultured with vehicle were set to 100%. Data are the mean of ± SEM of 3 independent experiments (***, P<0.001; **, P<0.01; *, P<0.05).

Fig. 4: Effect of Icer silencing on Atf3 expression. The 3T3-L1 undifferentiated cells were transfected with a control RNA duplex (si-GFP, open bar) or with si-ICER (filled bar). **a**) Nuclear proteins were prepared 48 h after transfection and subjected to western blotting analysis for the quantification of Icer and Atf3 levels. To control the specificity of the silencing the expression of Creb1 is shown. The figure shows the results of a representative experiment out of three. **b**) Quantification of the Atf3 level by quantitative real-time PCR. Total RNAs were prepared 48 h after transfection. The mRNA level of *Atf3* was normalised against the *Rplp0* and the expression levels from cells cultured with vehicle were set to 100%. Data are the mean of ± SEM of 3 independent experiments (**, $P<0.01$).

Fig. 1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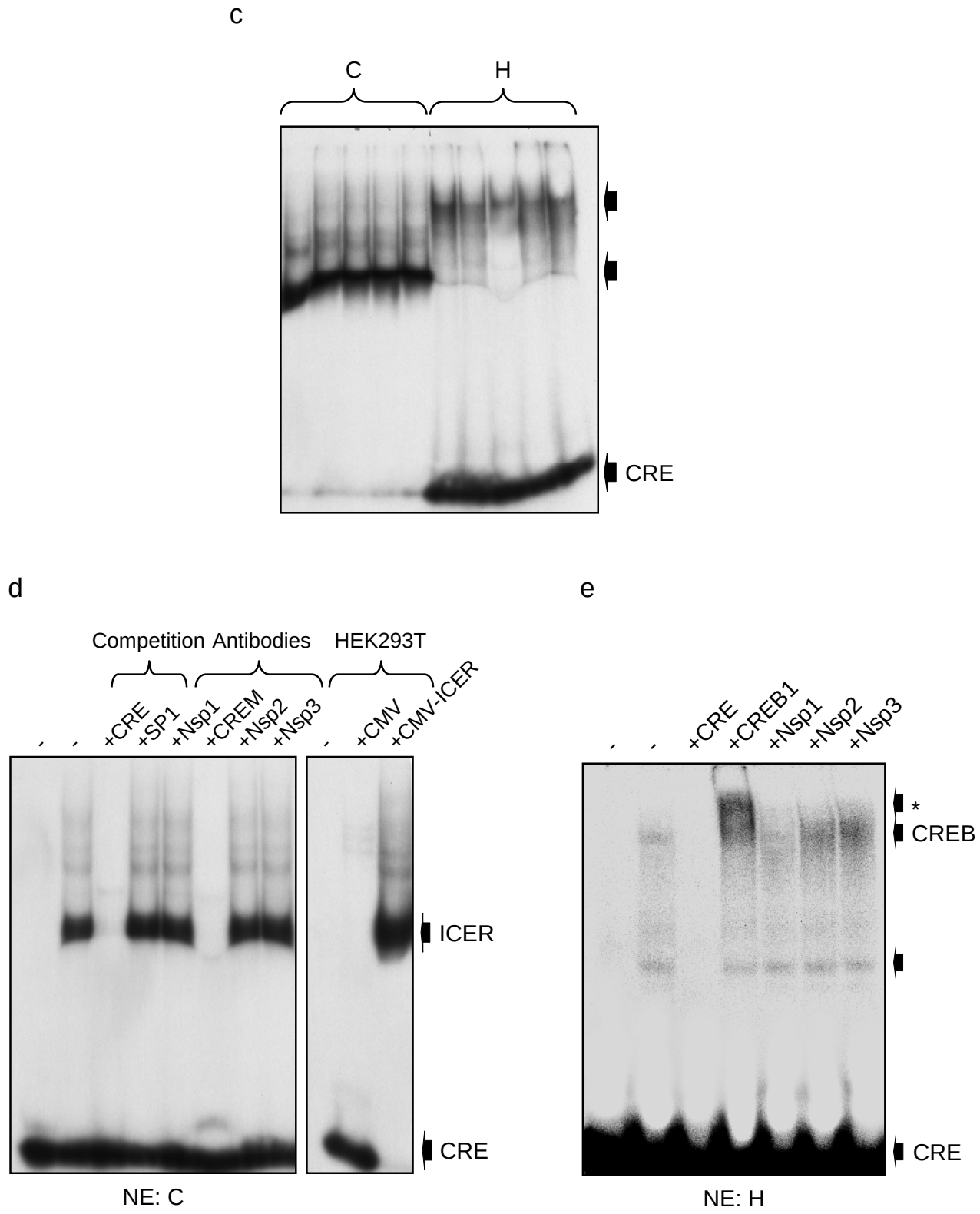
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Fig. 2

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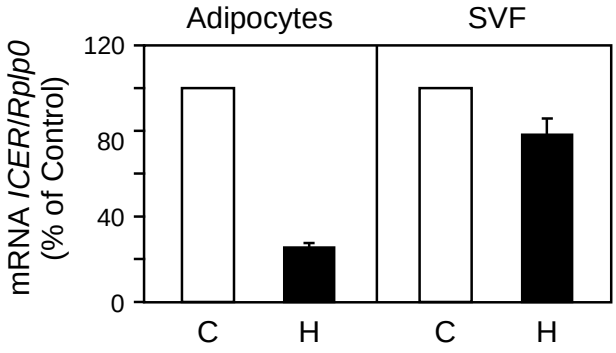
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mRNA ICER/Rplp0
(% of Control)
120
80
40
0
C
H
C
H

b

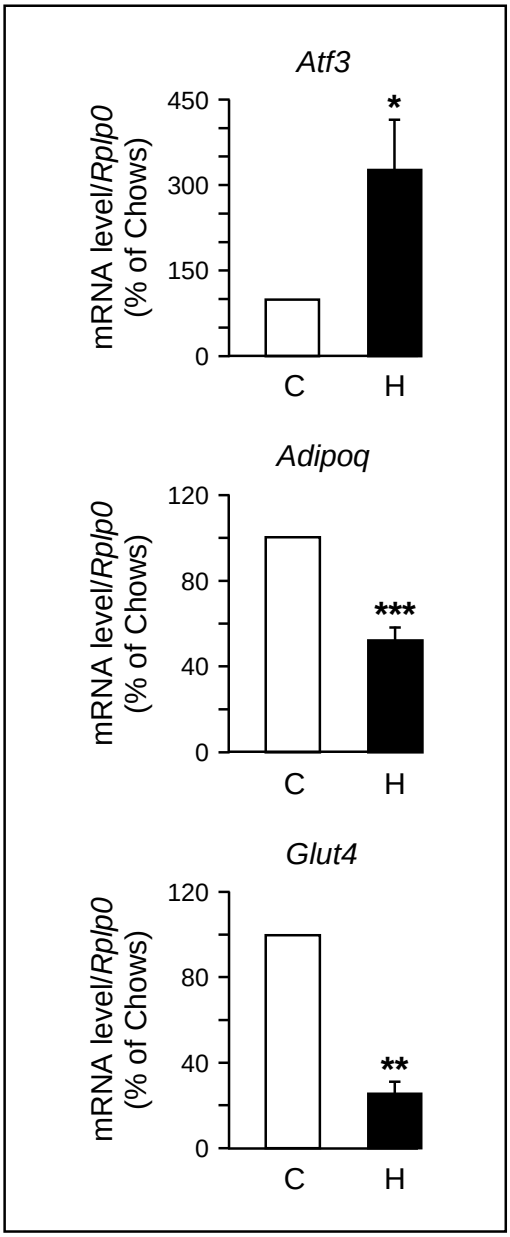

Atf3
mRNA level/Rplp0
(% of Chows)
450
300
150
0
*
C
H
Adipoq
mRNA level/Rplp0
(% of Chows)
120
80
40
0
***
C
H
Glut4
mRNA level/Rplp0
(% of Chows)
120
80
40
0
**
C
H

Fig. 3

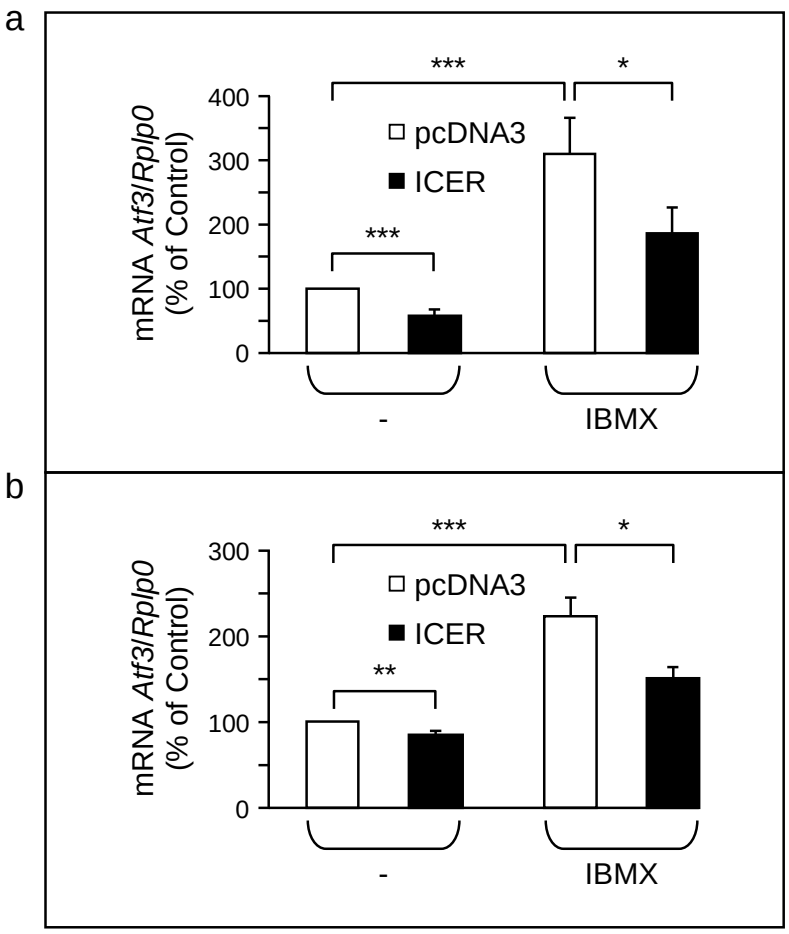
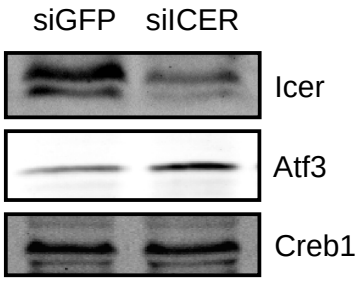
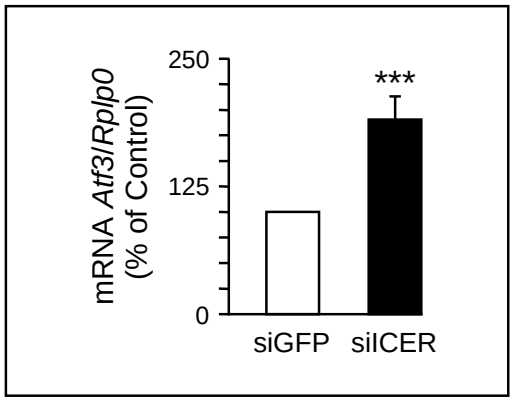

a
***
*
400
300
200
100
0
pcDNA3
ICER
***
mRNA Atf3/Rplp0
(% of Control)
-
IBMX
b
***
*
300
200
100
0
pcDNA3
ICER
**
mRNA Atf3/Rplp0
(% of Control)
-
IBMX

Fig. 4

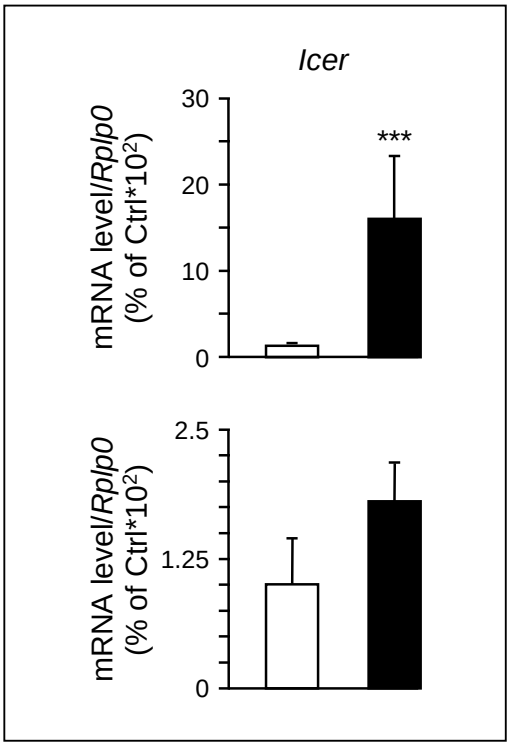
a


siGFP
siICER
Icer
Atf3
Creb1

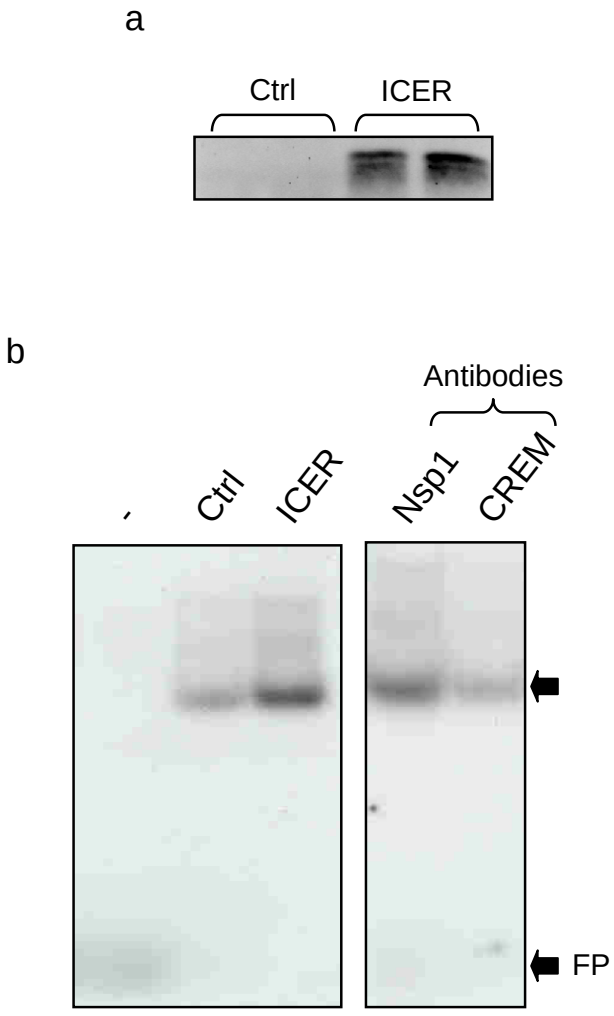
b


mRNA Atf3/Rplp0
(% of Control)
250
125
0
***
siGFP
siICER

Supplemental Fig. 1: Effect of the insulin on the expression of Icer in WAT. Insulin was infused in control (*upper panel*) and obese mice (*lower panel*) as described in supplemental methods. The animals were fasted for 10 h before hyperinsulinemic-euglycemic clamp. Total RNAs were prepared from WAT of control and obese mice that were infused with insulin (*filled bars*). The level of Icer was quantified by quantitative real-time PCR. The mRNA level were normalized against the *Rplp0* and the expression level from mice that were fasted for 10 h (*white bars*) was set to 100%. Data are the mean of ± SEM for 4 mice in each groups that were repeated 3 times (***, P<0.001).



Supplemental Fig. 2: Overexpression of Icer in 3T3-L1 adipocytes. **a**) The 3T3-L1 adipocytes were electroporated with the plasmid coding for Icer-Iγ (ICER) or with the empty vector (Ctrl). The expression of Icer was detected by Western blotting experiments using the anti-Crem antibodies. B) Measurement of Icer activity by EMSA. Nuclear protein extracts (NE) were prepared from 3T3-L1 adipocytes with either the empty vector (Ctrl) or ICER. NE were incubated with the CRE sequence as the labelled probe. To determine the binding activity of ICER, NE were pre-incubated with the anti-CREM (CREM) or the anti-HES-1 (Nsp1) antibodies as a negative control. The figures are representative of three independent experiments.



IV Discussion

IV Discussion

A Deleterious effects of oxidized LDL and protective action of HDL in β-cell: significance for β-cell dysfunction in T2D

Type 2 diabetes reaches nowadays epidemic proportions worldwide and is expected to affect more than 300 million people in the next 20 years, according to the World Health Organization. This disease become manifest when pancreatic β-cells fail to overcome insulin resistance by a compensatory hypersecretion of the hormone (14). Elucidating the mechanisms underlying β-cell failure is required to understand the molecular pathophysiology of T2D and to discover specific therapeutic strategies to block β-cell decline over time. In this respect, one of the objectives of this thesis was dedicated to shed light into the potential involvement of lipoproteins in β-cell dysfunction. The data obtained from *in vitro* experiments using insulin-producing cell lines and isolated islets, show that oxidized LDL induce a complete dysfunction of the β-cell, including a decrease in insulin expression, secretion and β-cell survival (56;80). On the other hand, as reported by several studies, 2mM of native LDL-cholesterol has no negative impact on β-cell function (56;80). However, higher doses of unmodified LDL exert adverse effects on β-cell survival and insulin release (55;87), revealing that supraphysiological concentrations of lipoproteins could hamper β-cell function in absence of oxidative modifications.

In macrophages, the class B scavenger receptors SR-B1 and CD36 are two important acceptors for the uptake of oxidized LDL (88;89). Peritoneal macrophages of CD36 null mice have a reduction of 70% of oxidized LDL binding, whereas the association of modified lipoproteins with cells overexpressing the SR-B1 receptor is massively increased (88;89). Interstingly, the

expression of these two scavenger receptors in macrophages is augmented in response to hyperglycemia and in diabetic animals (90;91). SR-B1 and CD36, as well as the LDL receptor which can recognize the mildly oxidized LDL (92), are present in pancreatic β-cells and could thereby mediate the harmful effects of modified lipoproteins ((55;56); Fig 1a and b, Appendix I). Other support in favor of a role for scavenger receptors in the harmful effects accomplished by oxidized LDL on β-cells could come from studies and observations performed on the glucagon-producing α-cells. While the α- and β-cells share similar islet environmental cues, cultured α-cells are, on the contrary, unable to uptake native LDL (57) and the expression of certain scavenger receptors such as SR-B1 and the LDL receptor is undetectable in these cells (Fig 1c, Appendix I). The notion that α-cells could be insensitive to toxic effects of oxLDL is further strengthened by the fact that hyperglucagonemia prevails with relative or absolute insulinopenia in plasma of patient with diabetes (93).

Another finding of our study is the protective role played by HDL-cholesterol. In line with this, we demonstrated that HDL particles efficiently counteract harmful outcomes on insulin synthesis and β-cell survival caused by oxidized LDL. Anti-apoptotic features of HDL have also been described on starvation-, cytokines- and hyperglycemia-mediated β-cell death, as well as on macrophage apoptosis evoked by oxidized LDL (55;87;94). Beneficial properties of HDL encompass efflux of cholesterol and oxysterol, antioxidant activity, oxidized phospholipids detoxification and anti-inflammatory capacity. Thus, all these observations strengthen the idea that low plasma levels of HDL might indirectly participate to β-cell failure by two mechanisms. The first one is the inefficiency of low levels of HDL in diabetes to counteract the effects of oxidized LDL, high glucose levels and cytokines on pancreatic β-cells. The second is the possible

incrimination of diminished antioxidant activity of HDL in the generation of oxidized LDL. Hyperglycemia generates glycated LDL that are sensitive to oxidization. Therefore low levels of HDL could decrease antioxidant activity and thereby, favor oxidization of LDL in diabetes. In turn, the modified particles would be responsible for the progressive decline of β-cells and development of atherosclerosis (Fig 4).

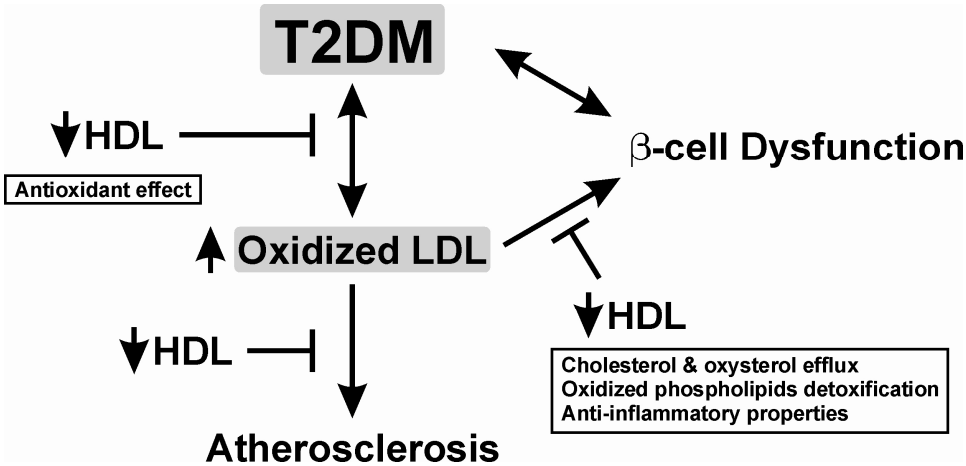


Fig 4: Deleterious effects of oxidized LDL and beneficial action of HDL in β-cell dysfunction and atherosclerosis

Future studies are needed to clarify whether HDL particles can exert their protective effect through receptor-mediated entry into the cells and/or by antagonizing process of stressors. The capacity of HDL particles to decrease both basal and IL-1β-induced pancreatic β-cell apoptosis supports the possible implication of these two mechanisms in HDL protection (87).

B Oxidative stress and ICER: two major players in β-cell dysfunction

Oxidized LDL have negative incidences on β-cells. Consequently, this prompted us to seek the molecular mechanisms involved. Activation of the JNK pathway has often been associated with the dysfunction of β-cells evoked by diabetic environmental factors, such as chronic hyperglycemia, elevated FFA and pro-inflammatory cytokines (78;95;96). Furthermore, modified lipoproteins trigger JNK pathway and AP-1 transcriptional activity in other cell types, ensuing in cellular apoptosis (81). The transcription factor c-Jun which constitutes part of the AP-1 complex, is activated by JNK and mediates the adverse effects of this signaling cascade on insulin expression and β-cell survival (97). In synergy with the activation of c-Jun, the decrease in the transcriptional activity of PDX-1 caused by the JNK pathway mediates loss of β-cell tasks in the face of chronic hyperglycemia (77). Induction of the JNK pathway elicits the export of the PDX-1 nuclear factor to cytosol and thereby, induces loss of insulin expression and cell survival (77). For these reasons, we therefore hypothesized a potential role for JNK pathway in the progression of β-cell dysfunction secondary to oxidized lipoproteins exposure. In agreement with this assumption we found an increase in JNK activity and c-Jun levels in β-cells cultured with oxidized LDL. The implication of this pathway in the alteration of β-cell function was illustrated by the restoration of insulin synthesis and cell survival by the JNK inhibitor peptide JNKi. We also found that the long-term JNK activity is inversely correlated with the expression of the JNK-interacting protein IB1. IB1 is an important negative regulator of JNK activity and is required for proper biosynthesis of insulin and β-cell survival (78;79). We speculate that the reduction in IB1 content permits the sustained induction of the JNK pathway by modified lipoproteins, the impairment in insulin biosynthesis and the initiation of programmed cell death. On the other hand, IB1 is not involved in the rapid activation of this signaling cascade, since the expression level of this protein is not affected after 45 minutes of exposure to oxidized LDL. This result

suggests that another process regulates the short-term triggering of JNK by modified lipoproteins. The mechanism accountable for the diminution in IB1 content is now elucidated and involves the transcriptional repressor ICER.

The expression of ICER is induced in response to the major environmental stressors related to T2D, including chronic hyperglycemia, hyperlipidemia and pro-inflammatory cytokines ((71); Fig 2, Appendix I). ICER is a basic leucine zipper transcription factor that negatively regulates the expression of numerous genes containing CRE sequences within their promoter (60;98). The lack of a transactivation domain makes ICER playing as a passive transcriptional repressor (61). Induction of this negative transcription factor by diabetic stressors has been reported to participate to the deterioration in insulin biosynthesis and secretion, and initiation of β-cell death (72-74). Similarly, we hypothesize that the transcriptional repressor ICER drives the adverse outcomes of the modified lipoproteins on pancreatic β-cells. Several data of the present thesis support this assumption. First, we found an induction of ICER in β-cells chronically exposed to oxidized LDL which was associated with dysfunction of these cells. Secondly, the selective reduction in ICER content prevented the impairment in insulin biosynthesis and secretion, and cell survival evoked by modified LDL.

On the one hand, the capacity of the transcriptional repressor ICER to inhibit the expression of the CRE-containing IB1 gene (Fig 6e, Appendix II) interferes with insulin production and β-cell survival. Indeed, reduction in IB1 content has been shown in this thesis to contribute to the loss of insulin biosynthesis and cell survival evoked by oxLDL. In addition, ICER could directly repress the transcription of insulin, owing to the presence of a CRE sequence in the promoter of this hormone (99). On the other hand, the sustained activation of ICER drives defects in glucose-

induced insulin secretion elicited by chronic hyperglycemia (73). In this context, impaired insulin release is in part linked to drop in the expression of at least four key genes governing insulin exocytosis, the GTPases Rab3a, Rab27a and their effectors Slp4 and Noc2, respectively (73). Similarly we observed a reduction in the levels of these four exocytotic genes in presence of oxidized LDL which was prevented by the deletion of the transcriptional repressor ICER. This illustrates the central role of the factor ICER in the insult of insulin secretion by various diabetic stressors.

It is worth mentioning that beside their ability to stimulate the expression of ICER, the majority of the environmental factors associated with T2D, such as elevated levels of glucose, FFA and cytokines, generate oxidative stress in pancreatic β-cells (43;48;100). Regarding oxidized LDL, many *in vitro* studies report production of ROS and oxidative stress in various cell types exposed to modified lipoproteins (81;101). Our data confirm the role of oxidative stress in the induction of ICER in pancreatic β-cells. First, oxidized lipoproteins triggered activation of the JNK pathway, a fact that represents a hallmark of oxidative stress (77). Second, incubation of β-cells with hydrogen peroxide, which directly generates oxidative stress, mimicked the effects of oxidized LDL and led to the induction of ICER. Last, modified lipoproteins markedly increased production of ROS in β-cells and the antioxidant NAC prevented the rise in ICER expression evoked by these particles. In endothelial and smooth muscle cells, oxidized LDL evoke production of ROS in a mechanism that involves some redox enzymes, such as NADPH oxidases (NOXs), endothelial nitric oxide synthase (eNOS) and complexes of the respiratory chain (102;103). Interestingly, in pancreatic β-cells, NOXs and enzymes of the electron transport chain have been reported to participate to the exacerbated production of ROS by diabetic stressors, such as high glucose, palmitate and cytokines (104;105). Future experiments are required to examine

the contribution of these enzymes in the generation of oxidative stress by oxidized LDL in β-cells.

Oxidative stress links oxidized LDL to induction in the expression of ICER. Under physiological conditions, the expression of this transcription factor is regulated by two mechanisms. The first one involves the transcriptional activators CREB. The alternative intronic promoter driving ICER transcription contains a cluster of four CRE elements, making this transcription factor a target gene of the CREB and CREM pathways (61). The hypothesis that increased CREB activity is involved in the induction of ICER provoked by oxLDL is unlikely because this activity is itself repressed by an elevated constitutive expression of the repressor. In addition, exposure of insulin-secreting cells to hydrogen peroxide resulted in the up-regulation of ICER transcript in absence of an increase in CREB phosphorylation (Fig 3a, Appendix I). The other mechanism that could participate to the augmented expression of ICER by the modified LDL engages the histone deacetylases (HDAC) activity. While HDAC promote inhibition of genes expression in most cases, they can stimulate transcription of certain genes, such as ICER. This positive regulation organized by HDAC requires the assembly of the preinitiation complex on ICER promoter, thus favoring the expression of the gene (106). Preliminary experiments performed in our laboratory suggest that oxidative stress promotes induction of the transcription factor ICER in a mechanism that necessitates HDAC activity. Elevation in ICER expression elicited by oxidative stress was completely blocked by the HDAC inhibitor trichostatin (Fig 3b, Appendix I). Further experiments are required to determine whether induction of ICER observed in cells challenged with most of diabetes stressors such as oxLDL, glucose, FFA and cytokines, correlates with an increase in HDAC activity. The expression of ICER is normally transient. After reaching a certain level, ICER inhibits its own expression through the CRE contained within its promoter.

The persistent elevated expression of ICER in response to oxLDL also suggests that the inhibitory feedback loop is defective. The role of the HDAC activity on such dysregulation will also be investigated in the future.

C A role for ICER in the sustained adipose CREB activity in obesity

The critical role of ICER in β-cells has prompted us to investigate an implication of this repressor in other cell dysfunction involved in the pathogenesis of diabetes. A reduced expression of GLUT4 and adiponectin in adipocytes accounts for loss of insulin sensitivity. Mice lacking expression of one of these genes specifically in the adipocyte exhibit development of systemic insulin resistance (37;107). The expression of GLUT4 and adiponectin are regulated by the transcription factor ATF3, which is a target of the CREB factors. ATF3 reduces the expression of GLUT4 and adiponectin probably by acting as a transcriptional repressor within the promoter of these two genes (76). In adipocytes of insulin-resistant and obese mice, the CREB activity and the abundance of ATF3 are increased (76). For this reason, we suggested that loss of ICER expression could cause the increase in CREB activity and subsequent loss of adipose function. In line with this hypothesis, we observed that altered expression of ATF3, GLUT4 and adiponectin was associated with a decrease in the abundance of ICER in adipose tissues of obese human and mice. *In vitro* experiments further confirmed the regulation of ATF3 by ICER. Overexpression of ICER in adipocytes lowered the mRNA content of ATF3, and consequently elevated the expression of GLUT4 and adiponectin (Fig 4a, Appendix I). Conversely, reduction in the ICER content by RNA interference augmented the transcript and protein levels of ATF3, and subsequently decreased the expression of GLUT4 and adiponectin (Fig 4b, Appendix I). These data therefore support the following model; Reduction in ICER content in obese adipocytes could permit CREB to occupy the CRE element located in the promoter of the ATF3 gene. Induction of

this transcription factor would, in turn, silence the expression of the glucose transporter GLUT4 and the insulin-sensitizer adiponectin. Future investigations will have to clarify the mechanism leading to impaired expression of ICER in obesity. Chronic hyperinsulinemia, pro-inflammatory cytokines and fatty acids contribute to defects in insulin signaling, glucose uptake and the expression of adipokines in adipocytes (14;23). Next experiments will be to determine whether these environmental stressors may diminish the expression of ICER.

The CREB and CREM activators regulate the expression of ICER. Diminution in ICER level is expected to elicit an increase in the transcriptional activity of the CREB and CREM activators. Involvement of this mechanism in the defective expression of ICER seems therefore very unlikely. A role for HDAC activity in the dysregulation of ICER is rather assumed. In support of this hypothesis, we observed that inhibition of HDAC activity in adipocytes by trichostatin hampers the expression of ICER (Fig 5a, Appendix I). This result led us to quantify the overall activity of HDAC in adipose tissue of obese human and mice. Measurements revealed that defect in ICER content is associated with a major reduction in HDAC activity (Fig 5b, Appendix I). A mechanism for adipose dysregulation of ICER in obesity should therefore consider involvement of HDAC activity.

Our work emphasizes that variations in the expression of ICER could contribute to pancreatic β-cell and adipocyte dysfunction in a mechanism involving HDAC activity (Fig 5). We think that counteracting the dysregulation of ICER could be proposed as a therapeutic target to combat development of diabetes

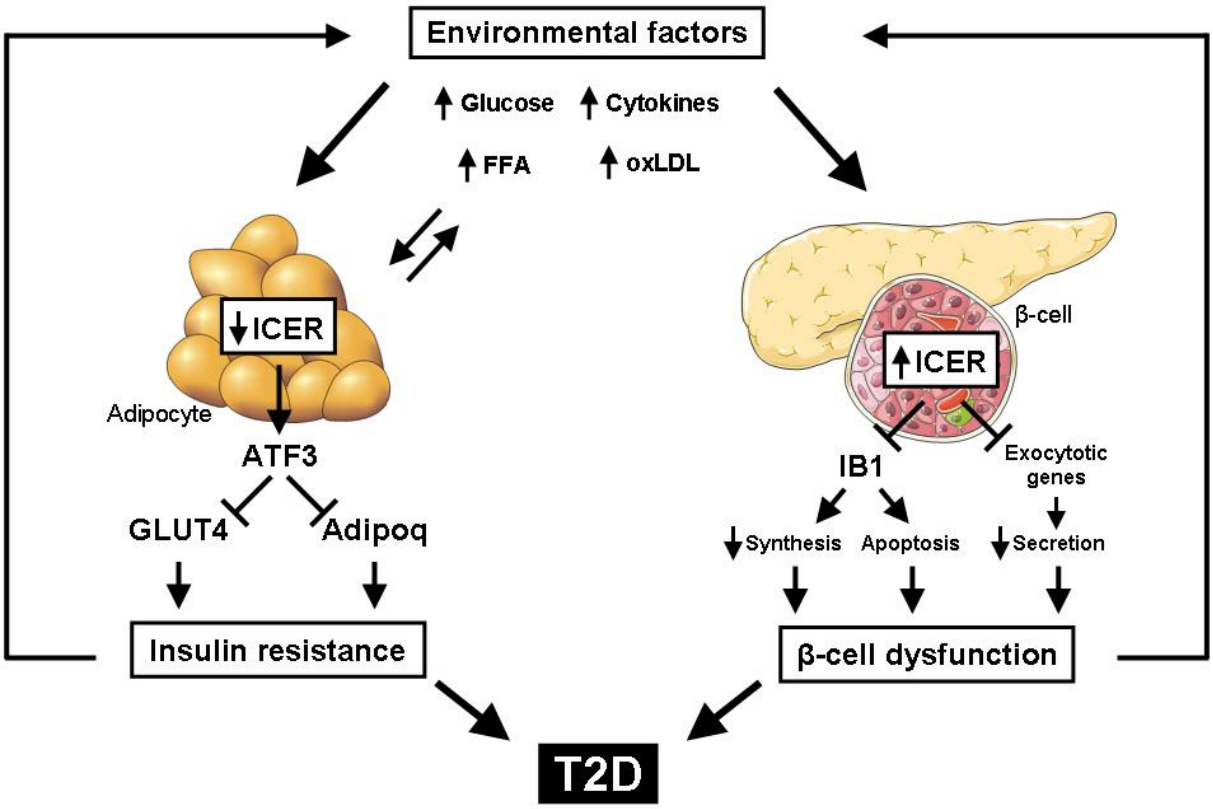


Fig 5: Role of the transcriptional repressor ICER in the molecular pathogenesis of type 2 diabetes

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V References

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Appendix I

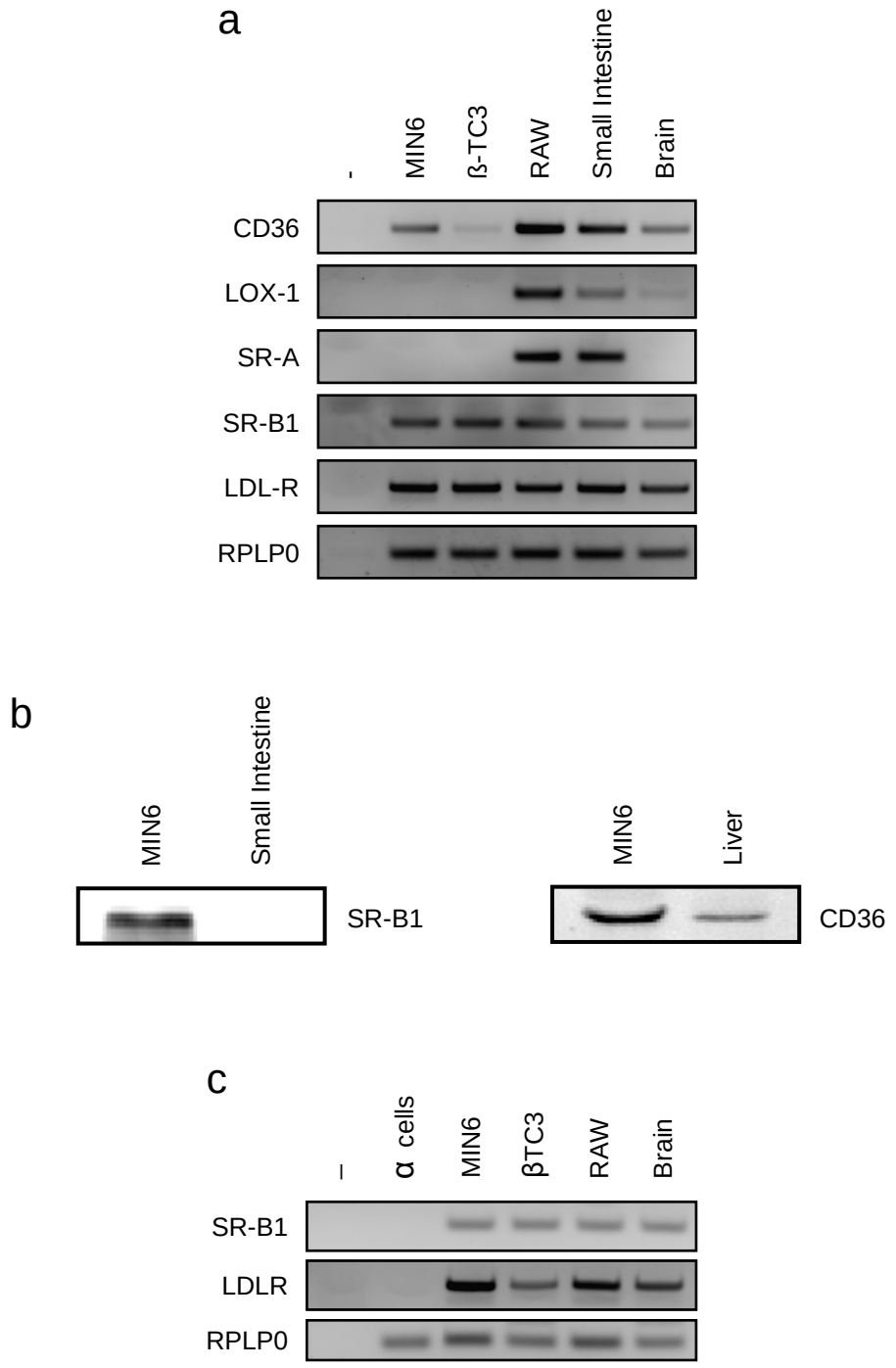

Fig 1: Expression of scavenger receptors in pancreatic α- and β-cells
(**a**) *CD36*, *LOX-1*, *SR-A*, *SR-B1*, *LDL-R* and *RPLP0* transcripts were amplified by PCR using cDNA that was prepared from MIN6, β-TC3 and RAW cells, and mouse small intestine and brain. (**b**) SR-B1 and CD36 proteins were detected in total protein prepared from MIN6 cells and mouse small intestine and liver by immunoblotting. (**c**) *SR-B1*, *LDL-R* and *RPLP0* transcripts were amplified by PCR using cDNA that was prepared from FACS-purified mouse α-cells, MIN6, β-TC3 and RAW cells, and mouse brain.

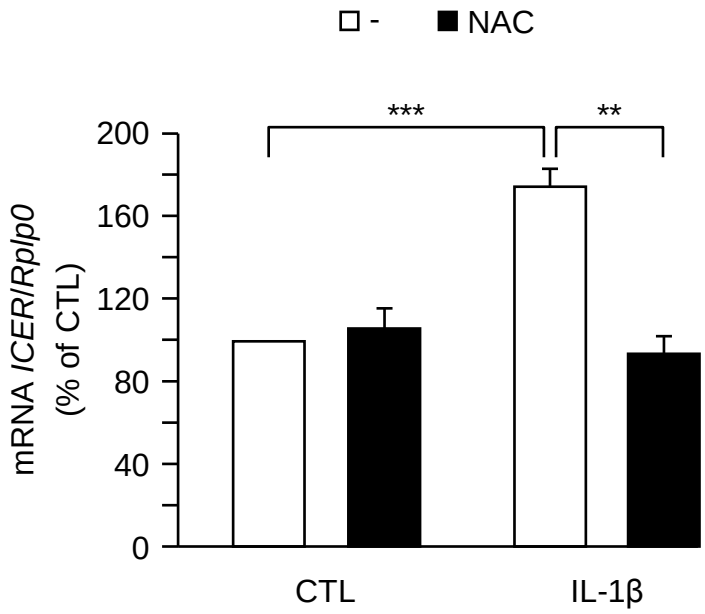

Fig 2: Role of oxidative stress in the activation of ICER by IL1-β
The mRNA level of *ICER* was quantified by quantitative real-time PCR using total RNA that was prepared from MIN6 cultured for 24h with 10ng/ml of IL-1β in the presence of vehicle (open bars) or NAC 1mM (filled bars). The mRNA levels were normalized against the *Rplp0* and the expression level from cells cultured with control (CTL) was set to 100% (***, P<0.001; **, P<0.01).

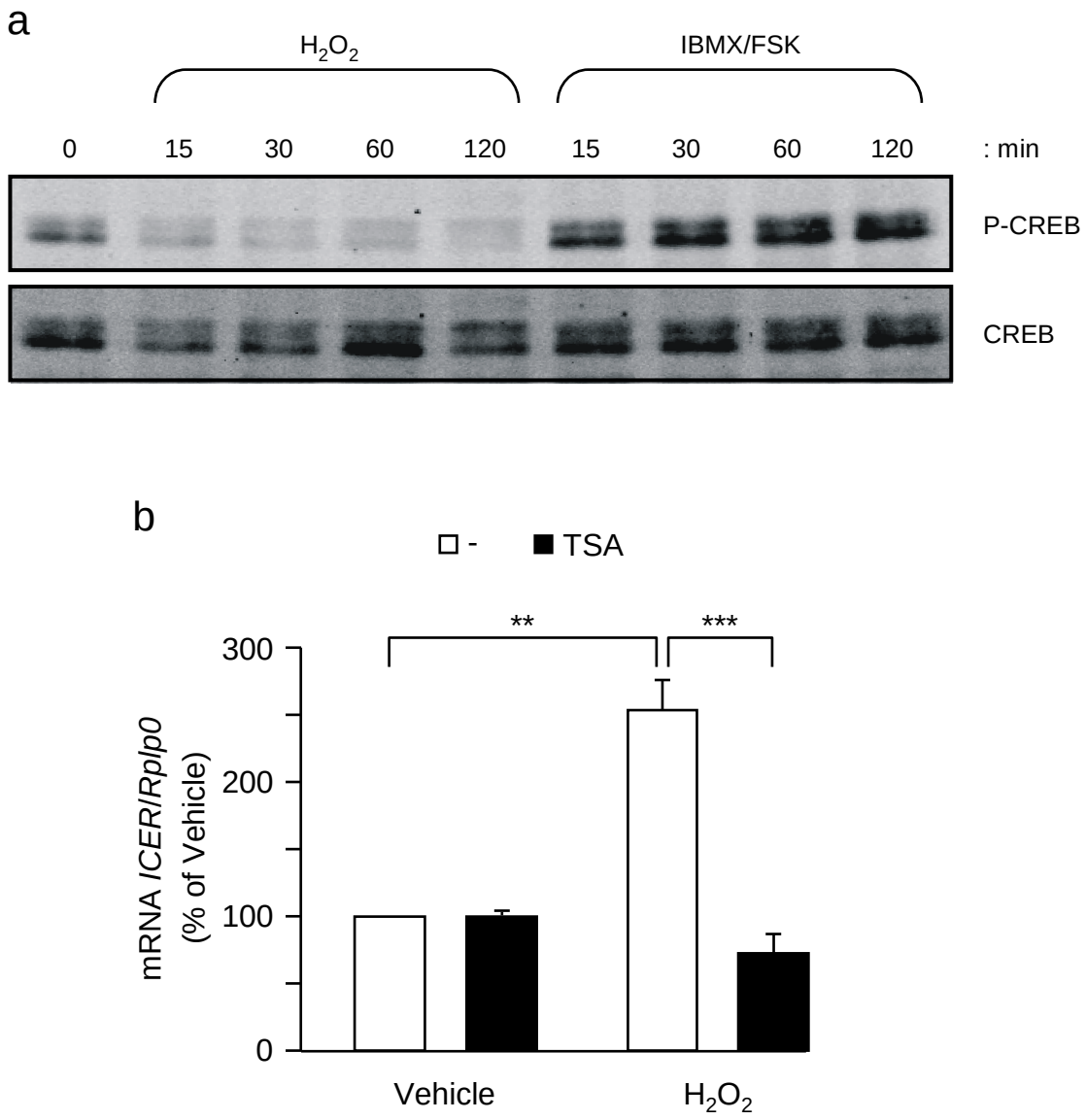

Fig 3: Role of histone deacetylase in the activation of ICER by oxidative stress
(**a**) Phosphorylated (P-CREB) and total CREB proteins were detected by immunoblotting in total protein prepared from MIN6 cells treated for 0 to 120 min in presence of 150µM of hydrogen peroxide (H_2O_2) or 100µM IBMX and 10µM Forskolin (IBMX/FSK). (**b**) The mRNA level of *ICER* was quantified by quantitative real-time PCR using total RNA that was prepared from MIN6 cultured for 2h with 150µM of H_2O_2 in the presence of vehicle (open bars) or trichostatin 10nM (TSA: filled bars). The mRNA levels were normalized against the *Rplp0* and the expression level from cells cultured with vehicle was set to 100% (***, P<0.001; **, P<0.01).

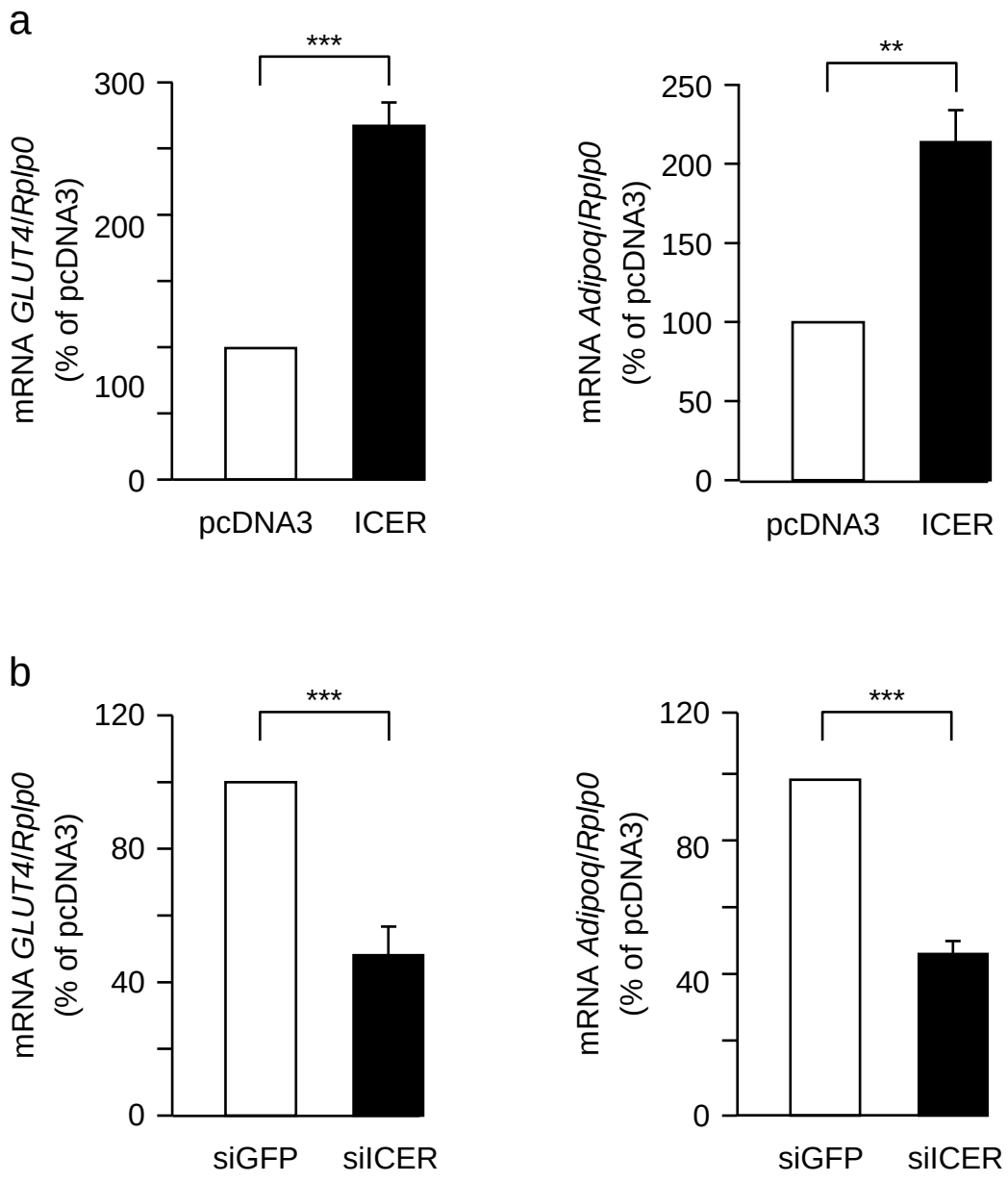

Fig 4: Effect of ICER on GLUT4 and adiponectin expression

(**a**) The mRNA level of *GLUT4* and *adiponectin (Adipoq)* were quantified by quantitative real-time PCR using total RNA that was prepared from 3T3-L1 adipocytes 4 days after electroporation with the empty vector (pcDNA3) or the plasmid coding for ICER .The mRNA levels were normalized against the *Rplp0* and the expression level from cells electroporated with pcDNA3 was set to 100% (***, P<0.001; **, P<0.01). (**b**) The mRNA level of *GLUT4* and *adiponectin (Adipoq)* were quantified by quantitative real-time PCR using total RNA that was prepared from 3T3-L1 fibroblastes 4 days after transfection with the control siRNA (siGFP) or the siRNA specific for ICER (siICER). The mRNA levels were normalized against the *Rplp0* and the expression level from cells transfected with siGFP was set to 100% (***, P<0.001).

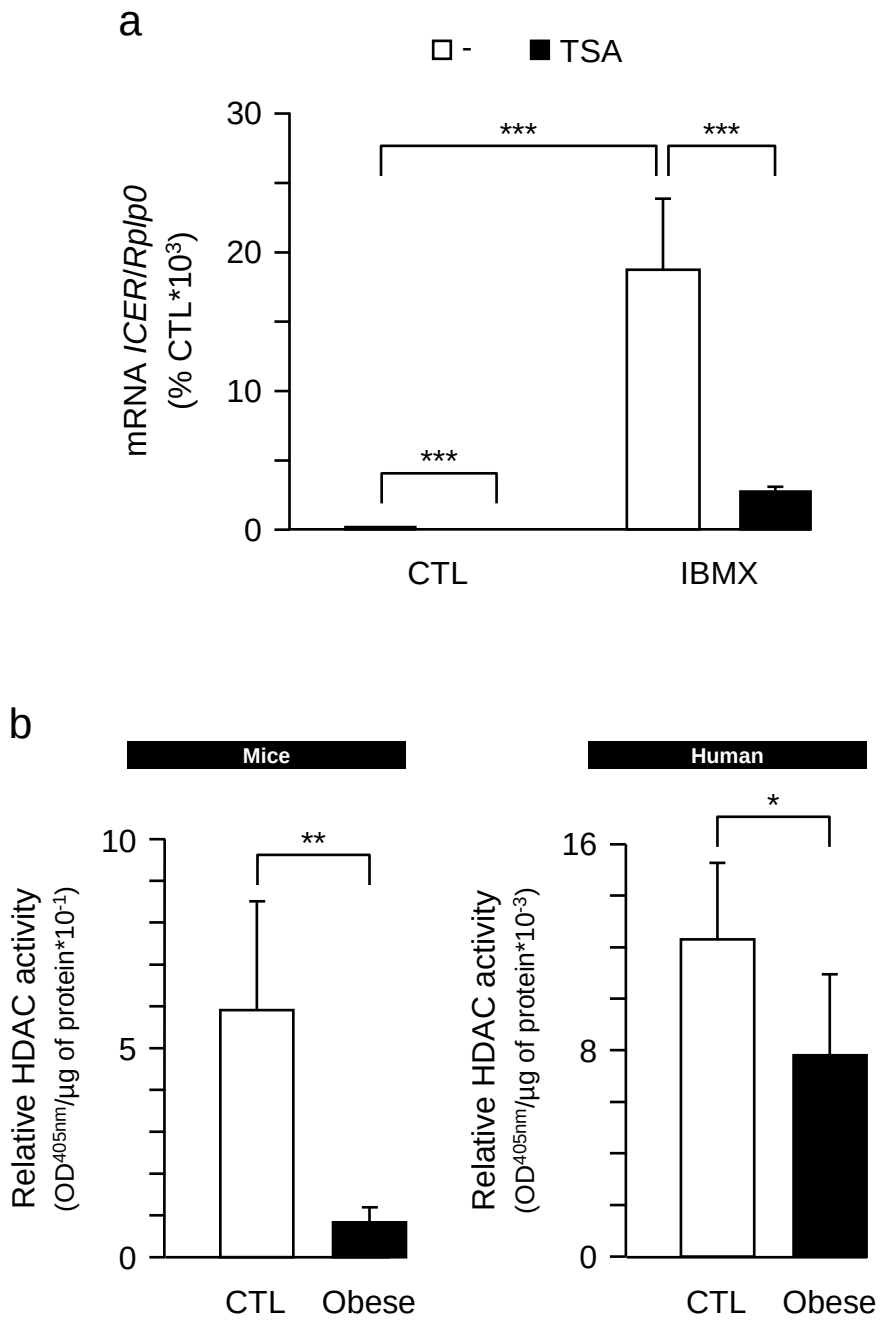

Fig 5: Role of histone deacetylase in the loss of ICER in obesity

(**a**) The mRNA level of *ICER* was quantified by quantitative real-time PCR using total RNA that was prepared from 3T3-L1 adipocytes cultured for 6h with 100µM IBMX in the presence of vehicle (open bars) or TSA 10nM (filled bars). The mRNA levels were normalized against the *Rplp0* and the expression level from cells cultured in control condition was set to 100% (***, P<0.001). (**b**) The total HDAC activity was quantified by ELISA using nuclear extracts prepared from white adipose tissue of control (CTL) and high fat diet-fed (obese) mice and visceral adipose tissue from lean (CTL) and obese human subjects. The HDAC activity levels were normalized against the protein content. (**, P<0.01; *, P<0.05).

Appendix II

Exendin-4 Protects β-Cells From Interleukin-1β–Induced Apoptosis by Interfering With the c-Jun NH_2-Terminal Kinase Pathway

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OBJECTIVE—The pro-inflammatory cytokine interleukin-1β (IL-1β) generates pancreatic β-cells apoptosis mainly through activation of the c-Jun NH_2-terminal kinase (JNK) pathway. This study was designed to investigate whether the long-acting agonist of the hormone glucagon-like peptide 1 (GLP-1) receptor exendin-4 (ex-4), which mediates protective effects against cytokine-induced β-cell apoptosis, could interfere with the JNK pathway.

RESEARCH DESIGN AND METHODS—Isolated human, rat, and mouse islets and the rat insulin-secreting INS-1E cells were incubated with ex-4 in the presence or absence of IL-1β. JNK activity was assessed by solid-phase JNK kinase assay and quantification of c-Jun expression. Cell apoptosis was determined by scoring cells displaying pycnotic nuclei.

RESULTS—Ex-4 inhibited induction of the JNK pathway elicited by IL-1β. This effect was mimicked with the use of cAMP-raising agents isobutylmethylxanthine and forskolin and required activation of the protein kinase A. Inhibition of the JNK pathway by ex-4 or IBMX and forskolin was concomitant with a rise in the levels of islet-brain 1 (IB1), a potent blocker of the stress-induced JNK pathway. In fact, ex-4 as well as IBMX and forskolin induced expression of IB1 at the promoter level through cAMP response element binding transcription factor 1. Suppression of IB1 levels with the use of RNA interference strategy impaired the protective effects of ex-4 against apoptosis induced by IL-1β.

CONCLUSIONS—The data establish the requirement of IB1 in the protective action of ex-4 against apoptosis elicited by IL-1β and highlight the GLP-1 mimetics as new potent inhibitors of the JNK signaling induced by cytokines. ***Diabetes*** **57:1205–1215, 2008**

Type 2 diabetes is characterized by a progressive decline in the number of insulin-producing β-cells and/or their intrinsic ability to produce and/or secrete insulin. With the classical treatment options of type 2 diabetes, a steady decline of β-cell function is observed, because none of the current treatment is aimed at the amelioration of β-cell deterioration. Toward the long-term improvement of β-cell mass, a new class of hypoglycemic mimetic agents and analogs of the glucoincretin glucagon-like peptide 1 (GLP-1) (1–5) offer a promising feature for patients with type 2 diabetes.

The anti-apoptotic role of GLP-1 has been determined in different β-cell models. GLP-1 and its long-acting receptor agonist, exendin-4 (ex-4) (6,7), increase the survival of immortalized rodent β-cell lines and purified rat β-cells when challenged with various pro-apoptotic stimuli, including the pro-inflammatory cytokine interleukin-1β (IL-1β) (8–13). The latter is thought to be implicated in the pathogenesis of type 2 diabetes. Increased levels of IL-1β and reduction in IL-1β–receptor antagonist content have been observed in pancreatic islets of patients with type 2 diabetes (14,15).

The GLP-1 promotes β-cell survival by interaction with GLP-1 receptor (GLP-1R), a member of the G_s-protein–coupled receptor superfamily (16). The mice with homozygous disruption for GLP-1R exhibit increased β-cell apoptosis in response to stress (12). Activation of GLP-1R, in turn, elevates cAMP levels and activates the protein kinase A (PKA) signal transduction system (9). PKA-dependent phosphorylation of the cAMP response element (CRE)-binding protein (CREB) stimulates the expression of various genes required for insulin secretion and cell survival (17,18). Disrupting CREB activity in islets causes β-cell apoptosis and diabetes in mice (18). Among the targets of CREB important for cell survival is the insulin receptor substrate 2 (18). The latter promotes islet-cell survival by mediating phosphorylation of Akt, also known as protein kinase B (PKB), in response to insulin and IGF-I signaling (19). The blockade of the PKB signaling partly prevents the protective effects of GLP-1 against cytokine-induced apoptosis (11).

Activation of the c-Jun NH_2-terminal kinase (JNK) pathway, a class of mitogen-activated protein kinases (MAPKs) also known as MAPK8, contribute largely to apoptosis of β-cells exposed to a stressful environment, such as cyto-

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ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE–binding protein; EMSA, electromobility shift assay; ex-4, exendin-4; GFP, green fluorescent protein; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; GST, glutathione S-transferase; HES-1, hairy and enhancer of split-1; IB1, islet-brain 1; IBMX, isobutylmethylxanthine; ICER, inducible cAMP early repressor; IL, interleukin; JBD, c-Jun NH_2-terminal kinase binding domain; JNK, c-Jun NH_2-terminal kinase; JNKi, JBD of IB1; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; RNAi, RNA interference; shIB1, target-specific shRNA directed against IB1; shRNA, short hairpin RNA; siRNA, small interfering RNA.

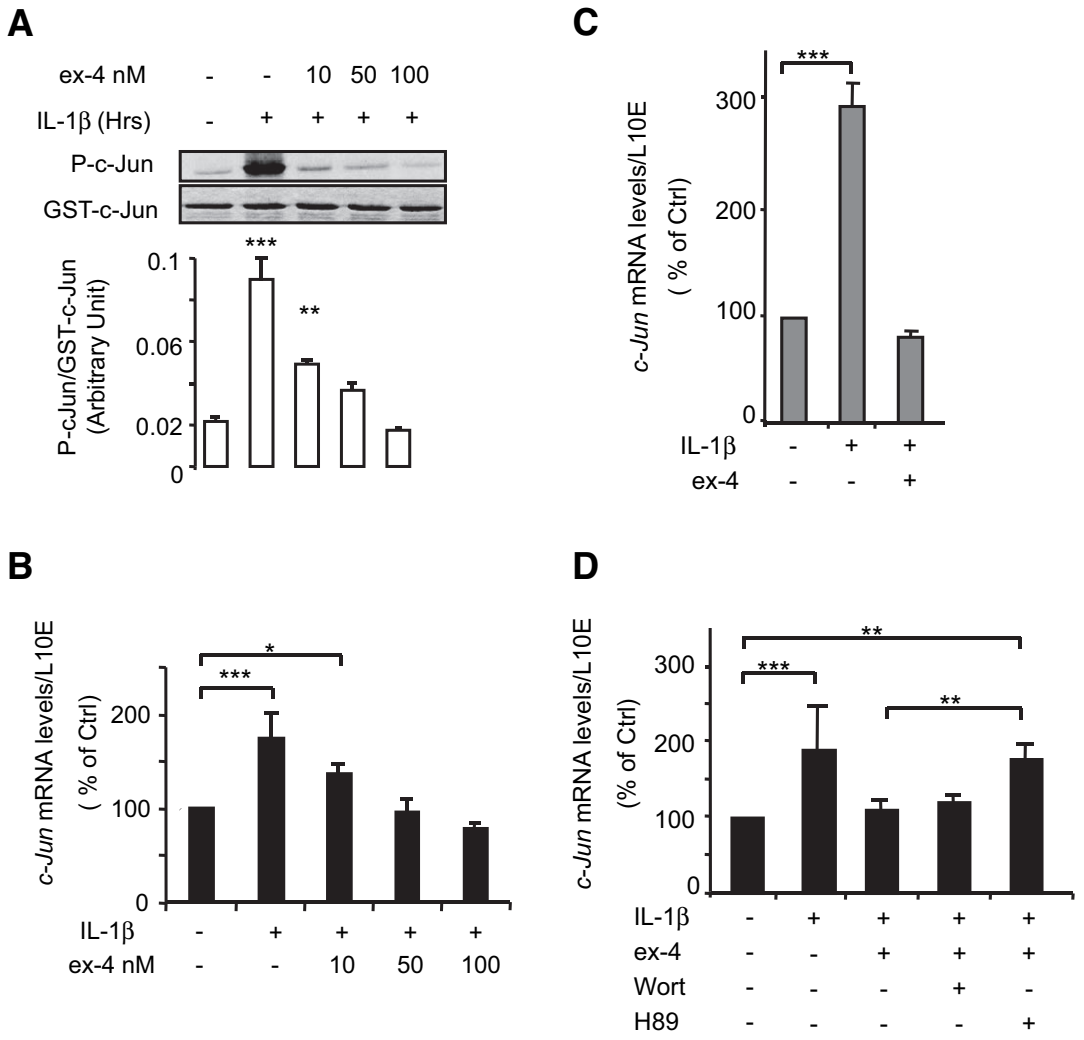

FIG. 1. Inhibition of IL-1β–induced phosphorylation and expression of c-Jun by ex-4. *A*: Effects of ex-4 on c-Jun phosphorylation. INS-1E cells were pretreated with ex-4 at indicated concentrations for 8 h. Thereafter the cells were exposed to 10 ng/ml IL-1β for 16 h. Solid-phase JNK assays were performed with the whole-cell lysates using GST–c-Jun as substrate. The reaction was loaded on a polyacrylamide gel, and γ-^{33}P phosphorylation of the substrates (P-c-Jun) was subsequently analyzed. The gel was stained with Coomassie blue to evaluate the loading of substrate (GST-Jun). The corresponding quantitation is depicted below the blots. The values correspond to the ratio in band intensities of phospho–c-Jun (P-c-Jun) over GST–c-Jun. Data are the means ± SE of four independent experiments. *$P < 0.05$; ***$P < 0.001$. The mRNA levels of *c-Jun* was quantified in INS-1E cells (*B*) and isolated rat islets (*C*) treated with ex-4 at indicated concentrations (or 100 nmol/l when not indicated) for 8 h followed by incubation with IL-1β for 16 h. The levels were normalized against L10E, and those of the untreated cells (Ctrl) were set to 100%. Data are the means ± SE of five independent experiments. *$P < 0.05$; ***$P < 0.001$. *D*: Effects of the PKA and PI 3-kinase/PKB inhibitors on the *c-Jun* expression. Total RNA was prepared from INS-1E cells that were cocultured with ex-4 (100 nmol/l) and IL-1β (10 ng/ml) in the presence of 10 nmol/l wortmannin (Wort) or 10 μmol/l H89. The expression of *c-Jun* mRNA was measured by real-time PCR and normalized against the housekeeping acidic ribosomal phosphoprotein P0 gene (L10E) gene. Data are the means ± SE of five independent experiments. **$P < 0.01$, ***$P < 0.001$.

kines (20). In most cases, activation of the JNK pathway is favored by the decline in expression of islet-brain 1/JNK-interacting protein 1 (IB1, now known as MAPK8 interacting protein 1), a scaffold protein that tethers components of the JNK pathway (21,22). The involvement of IB1 in the control of JNK activity and β-cell apoptosis is now well documented. A missense mutation (S59N) in the gene encoding IB1 (23) has been found in a French family with type 2 diabetes (23). Ex vivo, this mutation reduces the stability of IB1 leading to acceleration of the rate of cell apoptosis (23,24). Reduction in IB1 levels with the use of an adenovirus-mediated antisense cDNA leads to a concomitant increase in JNK activity and apoptotic rate (25). In contrast, overexpression of IB1 prevents IL-1β–mediated activation of JNK and renders the cells more resistant to apoptosis (25). IB1 interacts with JNK through JNK binding domain (JBD) (26,27). The delivery of a derivative peptide of the JBD linked to a HIV TAT is able to block JNK activation and fully protect β-cells against apoptosis induced by cytokines and oxidized LDL (21,26).

In this report, we demonstrate that ex-4 inhibits IL-1β–induced JNK signaling by stimulating the IB1 expression at the transcriptional levels. Suppressing the levels of IB1 with the use of RNA interference (RNAi) prevented the protective effects of ex-4 on β-cell apoptosis elicited by IL-1β. We provide evidence that the anti-apoptotic action of ex-4 against IL-1β–induced apoptosis involves inhibition of the JNK pathway.

RESEARCH DESIGN AND METHODS

Cell culture and preparation of pancreatic islets. Isolated human islets were from the Cell Isolation and Transplantation Center (islets for research distribution program) of the Geneva University Hospital. Islets were cultured in CMRL-1066 supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA) in 5% CO_2 humidified atmosphere at 37°C. The rat insulin-secreting

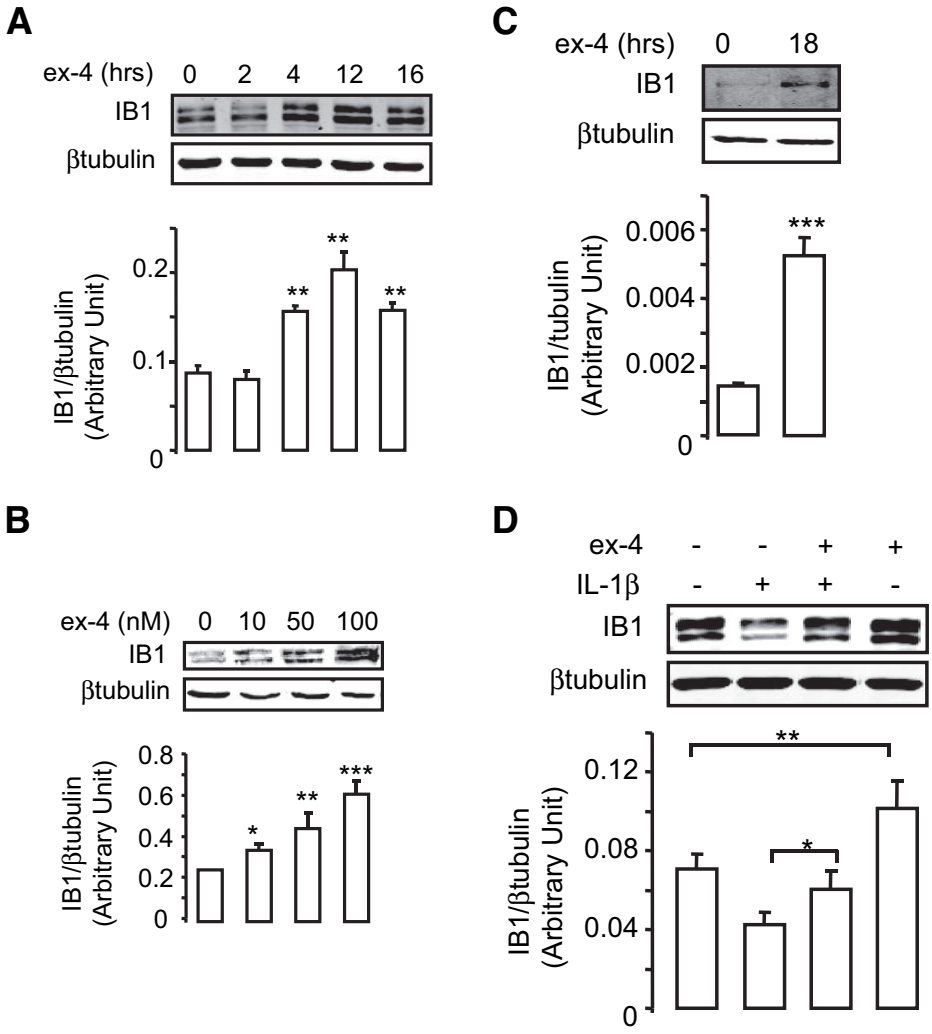

FIG. 2. Ex-4 stimulates the expression of IB1. Effects of ex-4 on the levels of IB1 in INS-1E cells exposed to ex-4 used at 100 nmol/l concentration at indicated times (*A*), at different indicated concentration for 12 h (*B*) and in isolated rat islets cultured with 100 nmol/l ex-4 (*C*) or INS-1E cell incubated with ex-4 plus 10 ng/ml IL-1β for 16 h (*D*). The levels of IB1 were assessed by Western blotting. The corresponding quantitation is depicted below the blots. The values correspond to the ratio in band intensities of IB1 over β-tubulin. Data are the means ± SE of four independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

cell line INS-1E was cultured as previously described (28). Rat and mouse islets were isolated by hand-picking after collagenase digestion of pancreas as described previously (29) and were maintained overnight in RPMI-1640 supplemented with 10% FCS, 10 mmol/l HEPES, pH 7.4, 1 mmol/l sodium pyruvate, 100 units/ml penicillin-streptomycin, 50 μmol/l β-mercaptoethanol, and 11 mmol/l glucose.

Western blotting. The cells were scrapped into the PBS and lysed using a passive lysis buffer (Promega). The cell protein extracts (20 μg) were separated on 10% SDS–polyacrylamide gel and electrically blotted to nitrocellulose membrane. The proteins were detected using a buffer containing 0.1% Tween 20 and 5% milk and incubated overnight at 4°C specific with primary antibodies (mouse IB1 antibodies [BD Transduction Laboratories] or mouse β-tubulin antibodies [Sigma]) and were visualized with IRDye 800 (Rockland) as secondary antibodies and quantified in an Odyssey Infrared Imaging System (Li-Cor). For quantification of IB1, we took the sum of the two band intensities and normalized against β-tubulin intensity.

Quantitative PCR. Total RNA was extracted using Aurum Total RNA mini kit (Bio-Rad) according to the manufacturer's protocol. The reverse transcription reaction was performed as previously described (30). Real-time quantitative PCR assays were carried out on the Bio-Rad MyiQ Real-Time PCR Detection System using iQ SyBr Green Supermix (Bio-Rad) as the amplification system with 100 nmol/l primers and 1.5 μl template (RT product) in 20-μl PCR volume and annealing temperature of 59°C. Primers sequences were as follows. Rat and mouse ib1: forward, 5′-ATGTCTTCATGAGTGGCCG-3′, and reverse, 5′-GATTTCAAGGACACAGCTGG-3; human IB1: forward, 5′-ATCAGCCTG GAGTTTGA-3′, and reverse, 5′-AGGTCCATCTGCAGCATCTC-3′; L10E (60S acidic ribosomal protein P0): forward, 5′-ACCTCCTTCTTCCAGGCTTT-3′, and reverse, 5′-ACCTCTTTCTTCCAAGCTTT-3; and rat *c-Jun*: forward, 5′-AGT CTC AGG AGC GGA TCA AG-3′, and reverse, 5′-CTC TGT CGC AAC CAG TCA AG-3′.

Promoter analysis, plasmids, and short hairpin RNA and small interfering RNA construction. The regulatory regions and promoter analysis of the human *MAPK8IP1* gene were subjected to a computer-assisted search (http://genomatix.de). To design target-specific short hairpin RNA (shRNA) directed against IB1 (shIB1), we selected sequences of the type $AA(N_{19})$ (N, any nucleotide) from the coding sequence of the rat IB1 mRNA. The selected small interfering RNA (siRNA) sequence was also submitted to BLAST search to ensure that it was specific to the target mRNA. Oligonucleotides contained both the 19-nucleotide sense and 19-nucleotide antisense strands separated with a short spacer from the reverse complement of the same sequence and five thymidines as termination signal. The primers used were the following: sense, 5′-GATCCCC CAGCGACTGGATTGACCAG **TT**CAAGAGA CTGGTCAA TCCAGTCGCTG TTTTTGGAAA-3′, and antisense, 5′-AGCTTTTCCAAAAA CAGCGACTGGATTGACCAG TCTCTTG**AA** CTGGTCAATCCAGTCGCTG GGG-3′. The complementary target sequences of IB1 and thymidines are underlined and in bold, respectively. These primers are hybridized and ligated downstream of the H1-RNA promoter by *Hin*dIII/*Bgl*II sites of the pSUPER vector (31). A 19-nucleotide prevalidated siRNA duplex (siIB1) that corresponds to the shIB1 sequence was designed as recommended and was chemically synthesized by Mycrosynth (Balgach, Switzerland).

Transfection and luciferase assays. The INS-1E cells (10^5) plated in 24-well dishes were transiently transfected using Effectene transfection reagent (Qiagen). After transfection (24 h), the cells were incubated with fresh medium supplemented or not with 10 μmol/l forskolin and 100 μmol/l

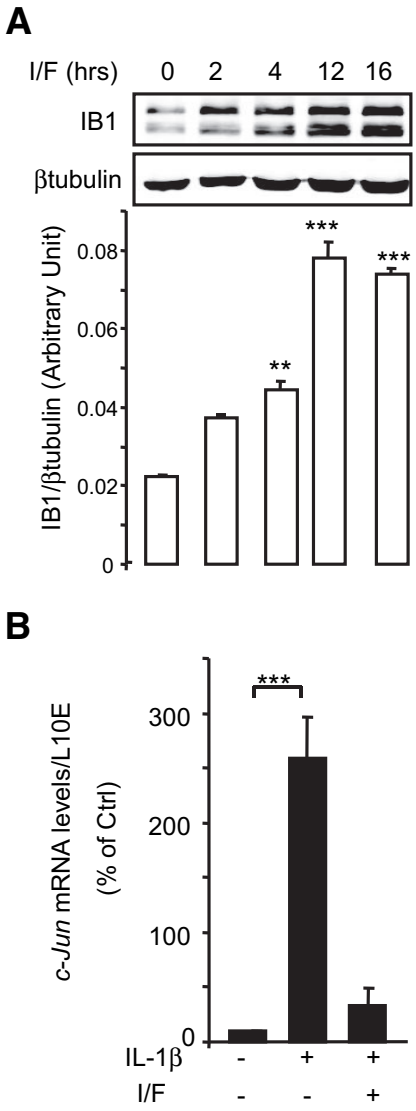

FIG. 3. The effects of ex-4 on IB1 expression is mimicked by cAMP-raising agents. *A*: INS-1E cells were cultured with 10 μmol/l forskolin and 100 μmol/l IBMX (I/F) at the indicated times. The expression of IB1 was analyzed by Western blotting. The corresponding quantitation is depicted below the blots. The values correspond to the ratio in band intensities of IB1 over β-tubulin. Data are the means ± SE of four independent experiments. **$P < 0.01$, ***$P < 0.001$. *B*: The expression of *c-Jun* was quantified in isolated rat islets treated with IBMX and forskolin and IL-1β for 16 h. The levels were normalized against L10E. Data are the means ± SE of five independent experiments. ***$P < 0.001$.

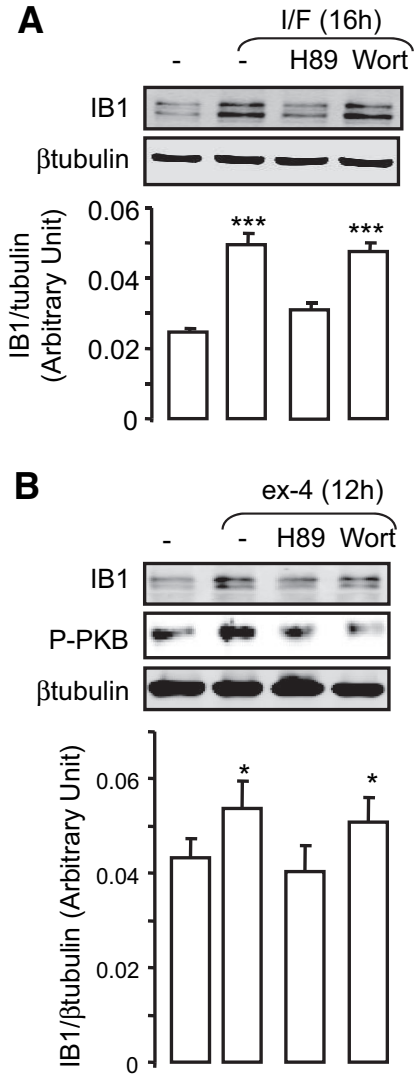

FIG. 4. Effects of the PKA and PI 3-kinase/PKB inhibitors on the IB1 expression. Western blotting analyses were performed to quantify the IB1 contents of INS-1E cells treated with IBMX and forskolin for 16 h (*A*) and ex-4 for 12 h (*B*), including 10 nmol/l wortmannin or 10 μmol/l H89, PI 3-kinase, and PKA inhibitors, respectively. Immunoblotting against phospho-PKB (P-PKB) was done to control the inhibitory effect of wortmaninn on the ex-4–stimulated PKB activation. The results are representative of three independent experiments. The corresponding quantitation is depicted below the blots. The values correspond to the ratio in band intensities of IB1 over β-tubulin. Data are the means ± SE of four independent experiments. *$P < 0.05$; ***$P < 0.001$.

isobutylmethylxanthine (IBMX) for 16 h. Then the cells were washed with PBS and lysed using passive lysis buffer (Promega). Luciferase activities were measured with 25-μl protein extracts solution using the Dual-Luciferase reporter assay system (Promega). The siRNA duplex was introduced using the lipofectamine 2000 (Invitrogen) exactly as described by the manufacturer's protocol.

Electromobility shift assay. Nuclear protein extracts from the cells were prepared exactly as described previously (32). The electromobility shift assay (EMSA) procedure was conducted exactly as previously reported (30). The sequences of oligonucleotides used are as follows. CreIB1: forward, 5′-TGTGTTACGTTACATT-3′, and reverse, 5′-AATGTAACGTAACACA-3′; CreL2: forward, 5′-GCTCTGAAGTCACTAA-3′, and reverse, TTAGTGACTTCAGAGC-3′; CreL3: forward, 5′-CCACTGACATCCTCTC-3′, and reverse, 5′-GAGAGGAT GTCAGTGG-3′; and CreCons: forward, 5′-GGACGTAGTCTGACGTCAGCGGA-3′, and reverse, 5′-CATCAGACTGCAGTCGCCTCCGA-3′. The sequences for specificity protein-1 (Sp-1) and neuron restrictive silencer element (NRSE) were those previously described (30).

Protein kinase assay. The preparation of whole-cell protein extracts and the kinase assays were conducted as previously described (21). Briefly, cell extracts were incubated for 1 h at room temperature with 1 μg glutathione S-transferase (GST)-Jun (amino acids 1–89) and 10 μl glutathione-agarose beads (Sigma-Aldrich, St-Gallen, Switzerland). Phosphorylation of substrate proteins was examined after overnight exposure of polyacrylamide gels to autoradiography; gel quantifications were accomplished by Phosphor-Imager analysis (Molecular Imager FX; Bio-Rad Laboratories, Basel, Switzerland).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were conducted exactly as previously described (33) with the following modifications. The anti-CREB1 and anti-CREB2 antibodies were used for immunoprecipitation. After the reverse cross-linkage, the DNA was precipitated by phenol-chloroform extraction method. Next, we performed the quantitative PCR to measure the enrichment of CreIB1 sequence by amplifying 5 μl DNA using the followings primers: forward, 5′-AGGTCTGCAGGGTTT GTCAT-3′, and reverse, 5′-CTGTGGTCTGCTGGGGTTAT-3′. Amplification of the *sytl4* promoter was used as an internal control: the primers used were forward, 5′-TGGGGGAGGGTATGGTAAAT-3′, and reverse, 5′-CCTTCTAG CACTCTGGAAGCA-3′.

Apoptosis assay. Apoptosis was determined by scoring cells displaying pycnotic nuclei visualized with Hoechst 33342 (Invitrogen, Basel, Switzerland) (21).

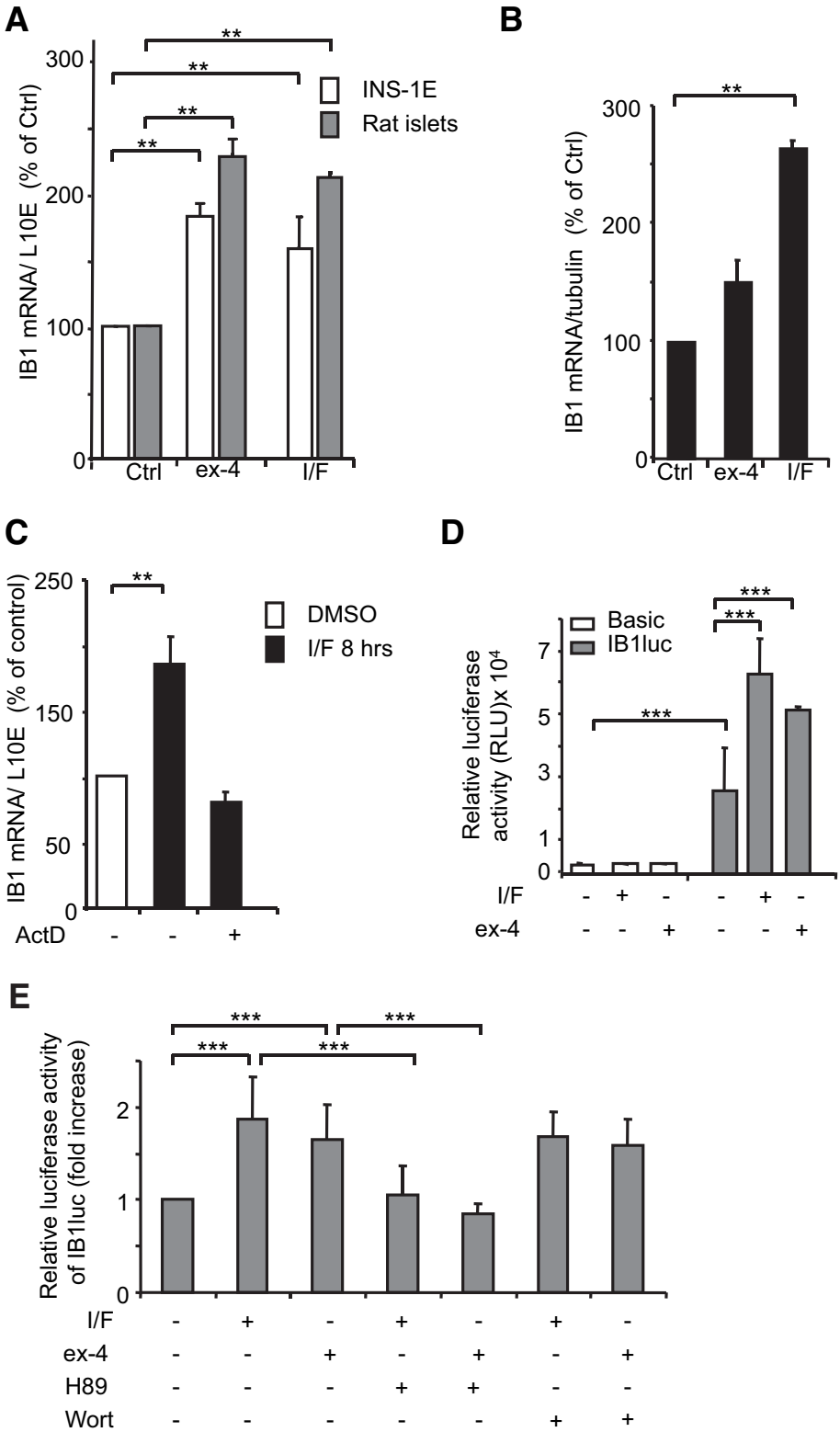

FIG. 5. Transcriptional regulation of IB1 by the cAMP/PKA pathway. Analysis of representative IB1 mRNA levels by real-time PCR from INS-1E (□) and rat isolated islets (▨) (*A*) and human isolated islets (■) (*B*) incubated with either ex-4 or 10 μmol/l forskolin and 100 μmol/l IBMX (I/F) for 16 h. The values obtained in untreated cells were normalized to 100%. The results are the means ± SE of three independent experiments performed in triplicate. ***P* < 0.01. *C*: Effects of actinomycin D on IBMX- and forskolin-stimulated IB1 expression. INS-1E cells were incubated with IBMX and forskolin for 8 h in the presence or absence of 5 μg/ml actinomycin D. ***P* < 0.01. *D*: Assessment of IB1 transcriptional activity in INS-1E cells cultured with IBMX and forskolin and ex-4. The cells were transiently transfected with a luciferase reporter construct driven by the 731-bp fragment of the human *MAPK8IP1* promoter (IB1luc). The cells were exposed to IBMX and forskolin or ex-4 for 16 h before measuring the luciferase activity. The data are the means ± SE of three independent experiments. ****P* < 0.001. RLU, relative light units. *E*: Effect of the PKA inhibitor H89 on the promoter activity of IB1. INS-1E cells were transiently transfected with the IB1luc construct and then incubated 24 h later after transfection with either IBMX and forskolin or ex-4 for plus or minus the PKA inhibitor H89 (10 μmol/l) or wortmannin for 16 h. All results from transfection experiments are the means ± SE of three independent experiments performed in triplicate. ****P* < 0.001.

Data analysis. Data are shown as means ± SE. Statistical significance of differences was calculated either by ANOVA or two-tailed *t* test for single comparisons.

RESULTS

Ex-4 interferes with IL-1β–mediated JNK signaling by modulating the expression of IB1. Exposure of β-cells to IL-1β mediates activation of JNK, which in turn induces phosphorylation of its target transcription factor c-Jun (26). This is observable between 1 to 16 h after incubation with IL-1β (22). To determine whether ex-4 could interfere with the JNK pathway, INS-1E cells were pretreated with ex-4 or the vehicle for 8 h, and thereafter, the cells were incubated with IL-1β for 16 h. Whole protein was then extracted from treated cells and incubated with the c-Jun recombinant. As expected, in vitro kinase assays showed an increase in c-Jun phosphorylation with extracts of cells incubated with IL-1β for 16 h (Fig. 1*A*). To validate

TABLE 1
The promoter of IB1 contains a Cre

	Species	Sequences
Consensus		TGACGTCA
CreIB1	Human	TTGTG**TTACGTTA**CATTC
	Rat	GGCTC**TGACGTTA**GGCA
	Mouse	CTTTT**TTACCTCAT**CTTG

The sequence was obtained by combining the consensus sequences. The putative binding elements identified in the promoters are shown in bold.

activation of the JNK signaling cascade by IL-1β, expression of the *c-Jun* gene was then quantified. The transcriptional activity of the promoter of this gene is positively regulated by JNK (21). Real-time PCR analysis showed a statistically significant augmentation by two- and threefold in *c-Jun* mRNA in INS-1E and rat isolated islet cells, respectively, cultured with IL-1β for 16 h (Fig. 1*B* and *C*). Both phosphorylation of c-Jun and its expression induced by IL-1β were efficiently diminished by ex-4 (Fig. 1*A*–*C*). The effect of ex-4 started at 10 nmol/l, but the maximum efficiency was obtained with 50–100 nmol/l concentration (Fig. 1*A* and *B*). To determine the pathways by which ex-4 inhibits the JNK signaling, INS-1E cells were co-incubated with wortmannin or H89, two pharmacological inhibitors of the phosphatidylinositol 3-kinase (PI 3-kinase)/PKB and PKA pathways, respectively. Quantitative PCR showed that inhibition of the PI 3-kinase/PKB signaling did not counteract the inhibitory effects of Ex-4 on the *c-jun* expression induced by IL-1β (Fig. 1*D*). In contrast, treatment of the cells with H89 prevented the inhibitory effects of ex-4 (Fig. 1*D*), indicating the requirement of the PKA activity for inhibition of the JNK signaling achieved in response to ex-4.

The scaffold protein IB1 has been described as a direct regulator of the JNK signaling. Overexpressing IB1 or its JNK-binding domain in β-cells prevents activation of c-Jun stimulated by IL-1β (25,26,34). We then assessed the possibility that ex-4 might modulate the levels of IB1 to block JNK-mediated activation of c-Jun. Western blotting experiments showed higher levels in IB1 contents in INS-1E cells incubated with 100 nmol/l ex-4 (Fig. 2*A*). The rise in IB1 protein levels elicited by ex-4 occurred in a time-dependent manner reaching a peak at 12–16 h (Fig. 2*A*) and was confirmed in isolated pancreatic islets from rat (Fig. 2*C*). Induction in the IB1 levels was observable with 10 nmol/l ex-4 but was further elevated with 100 nmol/l ex-4 for 12 h (Fig. 2*B*). In line with previous reports (22,34), we found that activation of the JNK signaling by IL-1β was associated with a reduction in the IB1 contents (Fig. 2*D*). Western blotting experiments showed that ex-4 efficiently counteracted the diminution of IB1 elicited by IL-1β (Fig. 2*D*). Taken together, these results indicate that ex-4 prevents activity of JNK by increasing the IB1 levels.

Ex-4 stimulates the expression of IB1 in a transcriptional mechanism requiring the PKA and CREB1 transcription factor. In view of the findings described above, we sought for a mechanistic explanation of the augmentation of IB1 elicited by ex-4. Because the latter regulates the expression of many genes through the cAMP/PKA pathway, we assessed whether the effect of ex-4 could be mimicked with cAMP-raising agents. Exposure of INS-1E cells with forskolin and IBMX led to an increase in IB1 protein levels (Fig. 3*A*). The increase in IB1 expression was apparent after a 2-h incubation and was maximal after 12–16 h (Fig. 3*A*). This result was consistent with an effect of IBMX and forskolin on JNK activity. Treatment of the isolated rat islets with IBMX and forskolin prevented induction of *c-jun* mRNA mediated by IL-1β (Fig. 3*B*). Incubation of INS-1E cells with H89 abolished the action of ex-4 and IBMX and forskolin on the IB1 expression, confirming that the effect of ex-4 involves the cAMP/PKA pathway (Fig. 4*A* and *B*). Induction in the IB1 levels evoked by ex4 was however unaltered in the cells co-treated with the PI 3-kinase/PKB pathway inhibitor wortmannin (Fig. 4*B*).

We then explored whether ex-4 modulates IB1 content by enhancing mRNA levels. Real-time PCR analysis showed an increase in IB1 mRNA in INS-1E and in isolated pancreatic islets cells from rat and human cultured for 12 h with ex-4 or IBMX and forskolin (Fig. 5*A* and *B*). Treatment of INS-1E cells with actinomycin D, a RNA synthesis inhibitor, prevented IBMX- and forskolin-mediated induction of IB1 (Fig. 5*C*), suggesting an effect of the cAMP pathway on IB1 at the transcriptional level. To test this hypothesis, a 731-bp fragment of the human IB1 proximal promoter linked to a luciferase gene reporter (IB1luc) (35) was transfected in INS-1E cells. A high luciferase activity from the IB1luc construct was detected in INS-1E cells as previously described (35). We found that incubation of the cells with IBMX and forskolin or ex-4 generated a twofold increase in the transcriptional activity of the IB1 promoter (Fig. 5*D*). To determine whether the transcriptional induction of IB1 requires the PKA activity, INS-1E cells were transfected with IB1luc and were treated with IBMX and forskolin plus or minus the H89 or wortmannin. Addition of the PKA inhibitor significantly diminished stimulation of the IB1 promoter activity mediated by IBMX and forskolin or ex-4 (Fig. 5*E*). Inspection of the human promoter revealed the presence of a CRE (CreIB1), which is homologous to the consensus sequence and conserved between rat and mouse (Table 1). EMSAs were performed to validate the ability of the CreIB1 sequence to interact with the CREB transcription factors. The CreIB1 sequence was used as the labeled probe and incubated with nuclear extracts of INS-1E cells treated with IBMX and forskolin for 16 h. The binding pattern obtained was efficiently competed using a 100-fold excess of the unlabeled CreIB1 and the consensus oligonucleotide (Fig. 6*A*). Neither the cold sequences of the IB1 promoter similar to the CreIB1 sequence (CreL2 and CreL3) nor the unrelated sequences, including Sp-1 and NRSE, were able to compete with the binding activity of CreIB1, confirming the high specificity of the probe/protein interaction (Fig. 6*A*). Preincubation of nuclear protein extracts with antibodies that recognize CREB1 or CREB2 factors resulted in a disruption of the CreIB1 pattern only with the CREB1 but not with the CREB2 or hairy and enhancer of split-1 (HES-1) antibodies (negative control) (Fig. 6*B*). After a rise in cAMP concentration, CREB transcription factors are rapidly phosphorylated by PKA (36). Phosphorylation of CREB increases the ability of the factors to bind to the target element, thereby converting CREB to a powerful activator (37). In line with the induction of the CREB binding activity, we observed an increase in the intensity of the CreIB1 binding pattern in cells cultured with IBMX and forskolin or ex-4 for 12 h (Fig. 6*C*). ChIP followed by real-time PCR experiments confirmed the in vivo interaction of CREB1 to the endogenous IB1 promoter in insulin-secreting cells. Enrichment of the PCR product for IB1

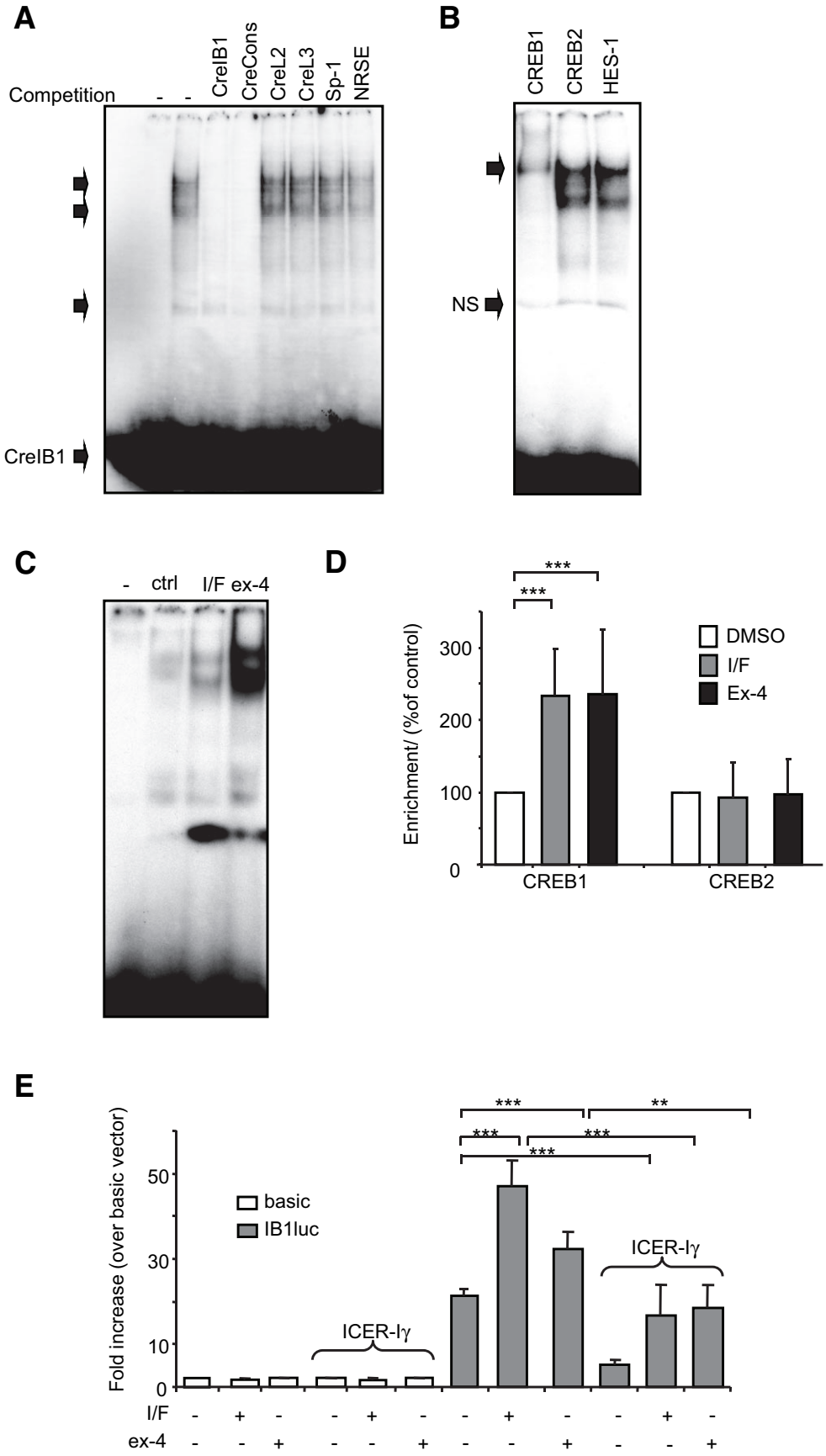

FIG. 6. CREB1 binds to the CRE element within the promoter of IB1. *A*: Nuclear extracts of INS-1E cells treated with 10 μmol/l forskolin and 100 μmol/l IBMX (I/F) were incubated with the radioactive probe containing the CreIB1 sequence. In the first lane, the nuclear extracts were omitted. Where indicated, the incubation was performed in the presence of 100-molar excess of unlabeled oligonucleotides corresponding to the CRE sequence of the human IB1 promoter (CreIB1), of the Cre consensus sequence (CreCons), or of the two Cre-like sequences found within the IB1 promoter (CreL2 and CreL3). In the last two lanes, competition was performed with the binding sequences for the Sp-1 and RE-1 transcription factors (NRSE). The radioactive complexes were analyzed by electrophoresis and visualized by autoradiography. The positions of the CREB complex and of the unbound probe are indicated. *B*: Before the addition of the labeled oligonucleotide, the nuclear extracts from INS-1E cells were preincubated with antibodies recognizing CREB1 (anti-CREB1), CREB2 (anti-CREB2), and the unrelated factor HES-1 (anti-HES-1) as negative control. The positions of CREB1 and of the unbound CRE probe are indicated. *C*: EMSA was performed with nuclear extracts from INS-1E cells treated with IBMX and forskolin or ex-4 for 16 and 12 h, respectively. The results are representative of three independent experiments. *D*: Occupancy of the promoter of IB1 by CREB1. Formaldehyde cross-linked chromatin samples from INS-1E cells incubated with or without IBMX and forskolin or ex-4 were used for immunoprecipitation reaction with anti-CREB1, anti-CREB2 and anti-HES-1 antibodies as a negative control. As a control of PCR, DNA aliquots from preimmunoprecipitated samples were used (data not shown). DNA was analyzed by PCR with specific primers corresponding to IB1. Amplification of the promoter region of *sytl4* that do not bind the CREB factors was used as negative and internal controls. The data obtained in untreated cells were normalized to 100%. ***$P < 0.001$. *E*: INS-1E cells were transiently transfected with the promoterless vector pGL3 basic (basic) (□) or IB1luc (▨). These plasmids were cotransfected either with an empty vector or with a plasmid leading to the overexpression of ICER-Iγ. Luciferase activities were measured 2 days later. The data are expressed as fold increase over the pGL3basic vector and are the means ± SE of three independent experiments. ***$P < 0.001$.

promoter was only detected with the use of immunoprecipitated DNA with the CREB1 antibody from the cells exposed to IBMX and forskolin or ex-4 (Fig. 6*D*). As observed in Fig. 6*B*, CREB2 did not associate with IB1 promoter (Fig. 6*D*). If CREB1 regulates the activity of the IB1 promoter, the transcriptional activity of IB1luc construct should be silenced by the inducible cAMP early repressor (ICER). ICER is a member of the CRE modulator family of basic leucine zipper transcription factors that is thought to serve as a dominant-negative repressor of cAMP-dependent gene expression (38). Overexpression of ICER-Iγ (28) reduced the basal and the IBMX and forskolin- or ex-4–stimulated promoter activity of IB1luc (Fig. 6*E*). In summary, these data reveal that ex-4 upregulates the expression of IB1 at the promoter levels in a mechanism involving the PKA and the CREB1 transcription factor.

Silencing in IB1 expression prevents the protective action of ex-4 against IL-1β–induced apoptosis. IB1 is an important component of pro-survival signaling pathway in β-cells. In this regard, we examined the possibility that the protective effect of ex-4 on β-cells might require IB1. To test this hypothesis, we used a RNAi strategy to selectively suppress the expression of IB1. First, we designed a plasmid expressing a specific and efficient sequence shRNA directed against IB1. INS-1E cells were then cotransfected with the HA-tagged IB1 (HA-IB1) and the plasmid encoding the shIB1. The specificity of the shRNA-IB1 was tested using a vector encoding HA-tagged IB1 resistant to the siRNA (HA-IB1-Si^r). Mutation was introduced within the target sequence by changing one nucleotide in each codon without affecting the primary sequence of the amino acids. The shRNA-IB1 efficiently reduced the expression of the exogenous HA-tagged IB1 (Fig. 7*A*). However neither the mutated IB1 construct, nor the green fluorescent protein (GFP) (cotransiently expressed) had their expression modified in the presence of the shRNA, confirming the specificity of the shRNA-IB1 (Fig. 7*A*). The effect of the siRNA on the endogenous levels of IB1 was assessed with a duplex siRNA sequence (siIB1) corresponding to the shIB1. Western blotting analysis of extracts from transfected INS-1E cells revealed the efficiency of the siIB1 to diminish the IB1 contents (Fig. 7*B*). Reduction in the IB1 levels led to an increase in basal c-Jun mRNA levels and potentiated the effects of IL-1β (Fig. 7*C*). However, ex-4 was unable to abolish induction of c-Jun achieved by IL-1β (Fig. 7*C*). To establish the involvement of IB1 in the anti-apoptotic action of ex-4, we therefore measured the viability of INS-1E cells in which IB1 was suppressed. As expected, incubation of the cells with 10 ng/ml IL-1β for 48 h led to a twofold increase in the rate of apoptosis, whereas the viability of the cells coincubated with ex-4 was unchanged compared with control (Fig. 7*D*). However, reduction in IB1 contents potentiated IL-1β–induced apoptosis and abolished the anti-apoptotic action of ex-4, confirming the requirement of IB1 in the mechanism of action of ex-4 (Fig. 7*D*).

DISCUSSION

The long-acting GLP-1R agonist ex-4 represents a new available class of drugs for treatment of type 2 diabetes. This disease is thought to occur when pancreatic β-cells fail to release sufficient insulin levels to compensate for insulin resistance in target tissues. Morphometric studies on postmortem pancreases of people with type 2 diabetes provide convincing evidence that β-cell mass is reduced, thus explaining in part the β-cell failure (39). Genetics and changes in environmental factors contribute to the β-cell destruction over time. These changes include an increase in concentration of IL-1β, a pro-inflammatory cytokine implicated in the pathogenesis of type 1 diabetes (14,15,40). IL-1β has detrimental effects in β-cells mainly through activation of the JNK signaling, which lead to increased phosphorylation and expression of its target c-Jun (26,41). In line with this statement, we showed a rise in JNK activity in the cells exposed to IL-1β, which was concomitant with increased β-cells apoptosis. In vitro and in vivo studies show that one of the major beneficial properties of ex-4 is the enhancement of β-cell mass at least through inhibition of apoptosis (8–13). In support of this function, we confirmed that pretreatment of the cells with ex-4 for several hours completely prevented apoptosis induced by IL-1β. Herein, we report that this effect of ex-4 is associated with diminution in phosphorylation and expression of c-*Jun* in isolated pancreatic islets and INS-1E cells challenged with IL-1β for 16 h. Inhibition of either the PKA or PI 3-kinase/PKB pathway, the two major pathways through which ex-4 triggers its anti-apoptotic effects, showed that only PKA is responsible for ex-4–mediated blocking of the JNK signaling. Incubation of the cells with IBMX and forskolin, two strong activators of the cAMP/PKA pathway, mimicked the effects of ex-4 on the *c-Jun* expression. Inhibition of PKB did not modulate *c-Jun* expression, whereas the use of H89, a pharmacological inhibitor of the PKA, also efficiently antagonized the effects of IBMX and forskolin on the JNK activity. We and others have previously shown that selective inhibition of the JNK pathway using the TAT-linked peptide derived from the JBD of IB1 (JNKi) reduces >50% of the β-cells apoptosis elicited by IL-1β, indicating that activation of the JNK pathway is one of the causes of IL-1β–induced apoptosis (41). In this report, we showed that the full protection mediated by ex-4 against apoptosis provoked by IL-1β was associated with an inhibition of the JNK pathway. Treatment of β-cells with a cocktail of cytokines (IL-1β, tumor necrosis factor-α, and interferon-γ) also leads to apoptosis through activation of JNK (11,42). However, in that case, ex-4 only confers a partial protection against apoptosis (11). This situation therefore assumes that inhibition of the JNK pathway is not sufficient for ex-4 to completely achieve its protective effect. A previous study reports that β-cells challenged with IL-1β alone had unaltered PKB activation (43) whereas phosphorylation of PKB is markedly reduced in response to cytokines. In the latter condition, ex-4 fails to recover the loss of PKB activation induced by cytokines (11). Thus it can be assumed that both inhibition of the PKA-dependent JNK signaling and adequate activation of PKB are needed for ex-4 to exert a full cytoprotective effect of β-cells against the cocktail of cytokines.

IB1, a scaffold protein that tethers components of the JNK pathway, is thought to facilitate the activation of JNK in acute response to various stimuli (44). Data obtained from different laboratories establish an important function for IB1 in the control of β-cell survival in response to stressful stimuli. Decreased IB1 expression caused by sustained exposure of β-cells to IL-1β or cocktail of cytokines, including IL-1β, tumor necrosis factor-α, and interferon-γ, is systematically accompanied by an increase in JNK-mediated phosphorylation of c-Jun (22,25,34). Consistent with this, we found that reduction in IB1 expres-

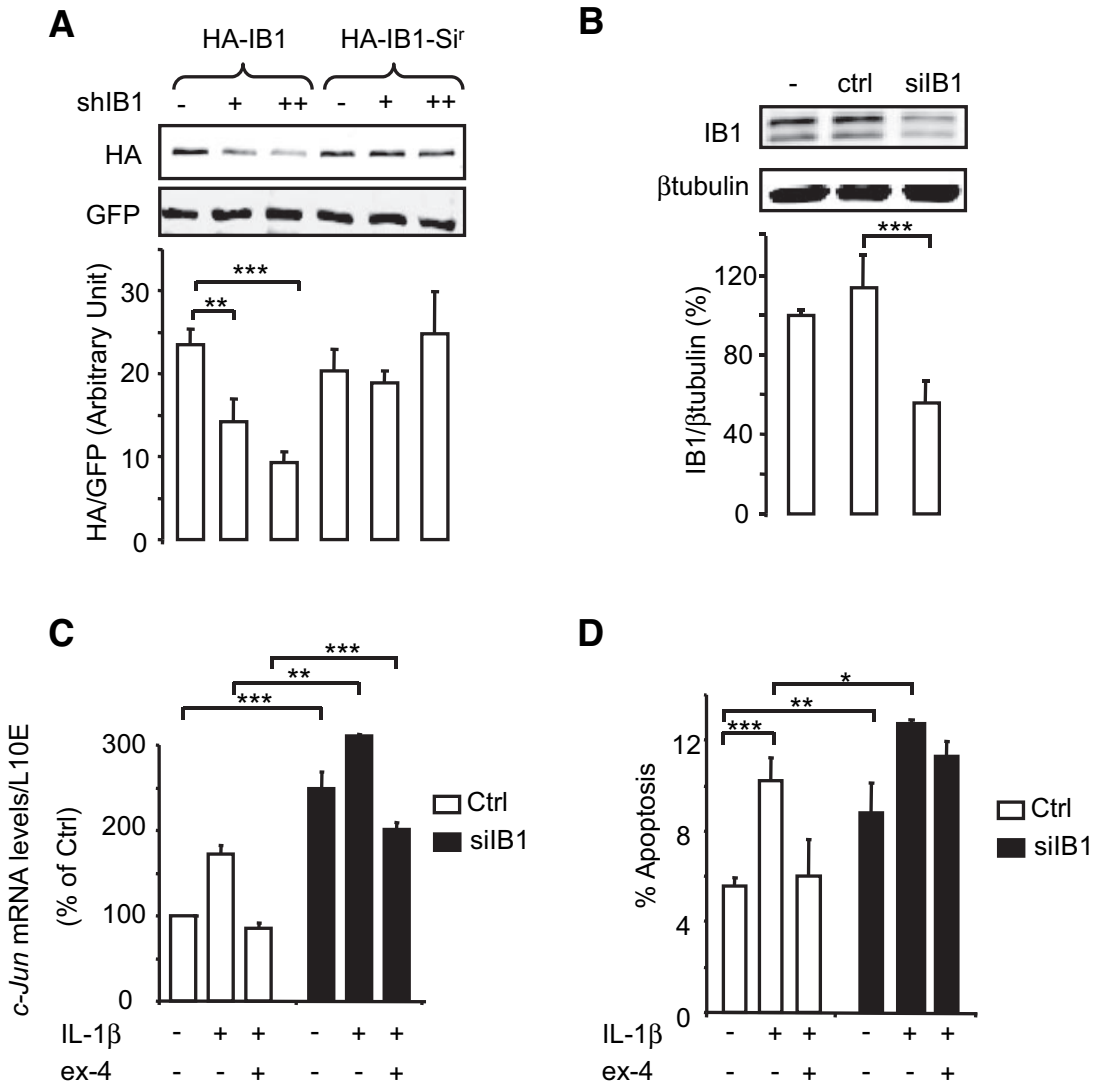

FIG. 7. Suppression of IB1 impairs the protective effects of ex-4. *A*: Inhibition of IB1 by RNAi. INS-1E cells were transiently cotransfected with 0.5 (+) and 1 μg (++) of the plasmids coding shIB1 and wild-type or shRNA-resistant HA-tagged IB1 (HA-IB1 and HA-IB1-Si^r, respectively) and a plasmid coding the GFP as a control of transfection efficiency. Two days later, total protein was extracted and submitted to Western blotting analysis. *B*: Inhibition of the endogenous expression of IB1 by a selected siRNA. INS-1E cells were transfected with the siRNA duplex directed against IB1 (siIB1) or scrambled siRNA (Ctrl). Results on the graph below the blot correspond to the fold changes observed in Western blot band intensities obtained from lysates of transfected cells with siRNAs. The values correspond to the ratio in band intensities of IB1 over β-tubulin. Data are the means ± SE of four independent experiments. **$P < 0.01$; ***$P < 0.001$. *C* and *D*: Effects of the IB1 silencing in the expression of c-Jun and apoptosis. INS-1E cells were transfected with siIB1 (■) or control siRNAs (□). Thereafter, the cells were preincubated 24 h after transfection with 100 nmol/l ex-4 for 8 h. The expression of *c-Jun* mRNA and rate of apoptosis were scored in ex-4–pretreated INS-1E cells exposed to IL-1β for 16 and 48 h, respectively. The c-Jun levels were normalized against the L10E gene. Data are the means ± SE of five independent experiments. Each experiment was performed three times in triplicate. Results are expressed as means ± SE of four independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

sion in the cells exposed to IL-1β was associated with more phosphorylated c-Jun and apoptosis. In addition, the latter phenotype was exacerbated in the cells where the resting IB1 levels were silenced with the use of the RNAi approach. Both apoptosis and the JNK activity can be efficiently overcome by compensating the reduced levels of IB1 in overexpressing IB1 or overloading β-cells with a JNKi (25,26,41). In this report, we show that the relative recovering of the IB1 levels after treatment of the cells with ex-4 for 12–16 h is also sufficient to reduce phosphorylation/expression of c-Jun and to abolish apoptosis elicited in response to IL-1β.

The mechanism leading to restored IB1 levels was deciphered. In fact, incubation of the cells with the PKA inhibitor H89 prevented the stimulation of the IB1 expression levels achieved by ex-4. Our data showed that inhibition of PKB activation does not interfere with the capacity of ex-4 to stimulate IB1 expression, indicating that PKB is not required for the effects of ex-4 on the JNK signaling. Using a combination of approaches, we demonstrated that ex-4 compensates IL-1β–mediated reduction of IB1 by stimulating the promoter activity of IB1 through the CREB1 transcription factor. The peak for IB1 induction was reached at 16 h. After that time, IB1 expression returned to the level comparable with that observed in untreated cells (data not shown). This mechanism could be attributed to the transcription factor ICER. After a sustained stimulation of the cAMP/PKA pathway, the transcriptional activation of the CREB factors is followed by the activity of ICER, a powerful repressor that negatively regulates expression of genes containing a CRE element (21). In agreement with a regulation of IB1 by

ICER, we found that overexpression of ICER-Iγ efficiently silenced the promoter activity of IB1. Suppression of IB1 expression by RNAi impaired the anti-apoptotic effect of ex-4. These data provide evidence that IB1 plays a key role in mediating the effect of ex-4 against apoptosis in a JNK-dependent manner. According to these data, defects in the GLP-1 signaling are expected to impair induction of IB1. Interestingly mice with targeted disruption of the GLP-1R have reduction in pancreatic β-cells mass caused by increased stress-induced apoptosis (12). Low plasma levels in GLP-1 have been observed in human type 2 diabetes (45). Therefore impaired induction of IB1 could partly account for elevated β-cell apoptosis in diabetes.

Activation of the JNK signaling is partly responsible for the loss of functional pancreatic islet β-cell mass after grafting. This loss of viability is caused by the pro-inflammatory cytokines, including IL-1β, and the islet isolation procedure itself with a major loss occurring shortly after the final purification steps (41,46–48). A recent report shows that improvement of β-cell viability is associated with a decreased JNK activity both in cultured rat and human isolated islets (46). Thus, direct inhibition of the JNK signaling cascade has been proposed as a strategy to improve the cell viability for transplantation purpose (46). To this end, we believe that ex-4 or long-acting analogs mimetics may be used as efficient available therapeutic drugs. Like IL-1β, prolonged exposure of β-cells to high glucose and nonesterified fatty acid concentration have devastating effects on β-cells and thereby participate in progression and development of type 2 diabetes. The mechanism responsible for β-cell failure include increased apoptosis that is associated with a rise in JNK activity (21,49,50). Ex-4 can also protect β-cells against apoptosis provoked by elevated concentrations of glucose and lipids (8). Thus it is possible that cytoprotective action of ex-4 against chronic hyperglycemia and hyperlipidemia involves inhibition of the JNK signaling.

This study highlights the potent inhibitory effect of ex-4–cAMP–PKA system on the JNK activity as a major mechanism for preventing β-cell apoptosis induced by IL-1β. This mechanism largely contributes to the efficiency of ex-4 and its mimetics analogs in the long-time preservation of β-cells against apoptosis in the treatment of type 2 diabetes.

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