Involvement of MicroRNAs in the Cytotoxic Effects Exerted by Proinflammatory Cytokines on Pancreatic β-Cells

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OBJECTIVE—Pancreatic β-cells exposed to proinflammatory cytokines display alterations in gene expression resulting in defective insulin secretion and apoptosis. MicroRNAs are small noncoding RNAs emerging as key regulators of gene expression. Here, we evaluated the contribution of microRNAs to cytokine-mediated β-cell cytotoxicity.

RESEARCH DESIGN AND METHODS—We used global microarray profiling and real-time PCR analysis to detect changes in microRNA expression in β-cells exposed to cytokines and in islets of pre-diabetic NOD mice. We assessed the involvement of the microRNAs affected in cytokine-mediated β-cell failure by modifying their expression in insulin-secreting MIN6 cells.

RESULTS—We found that IL-1β and TNF-α induce the expression of miR-21, miR-34a, and miR-146a both in MIN6 cells and human pancreatic islets. We further show an increase of these microRNAs in islets of NOD mice during development of pre-diabetic insulitis. Blocking miR-21, miR-34a, or miR-146a function using antisense molecules did not restore insulin-promoter activity but prevented the reduction in glucose-induced insulin secretion observed upon IL-1β exposure. Moreover, anti–miR-34a and anti–miR-146a treatment protected MIN6 cells from cytokine-triggered cell death.

CONCLUSIONS—Our data identify miR-21, miR-34a, and miR-146a as novel players in β-cell failure elicited in vitro and in vivo by proinflammatory cytokines, notably during the development of peri-insulitis that precedes overt diabetes in NOD mice. ***Diabetes*** **59:978–986, 2010**

Insulin release from pancreatic β-cells is essential for blood glucose homeostasis. Diabetes develops if the amount of insulin released by β-cells is insufficient to cover the metabolic demand. Type 1 diabetes is an autoimmune disease characterized by an inflammatory response against pancreatic islets, leading to selective and progressive β-cell loss (1). Type 2 diabetes, the most common form of the disease, is often associated with obesity and results from defects in insulin secretion or from diminished sensitivity of target tissues to insulin action (2). Although the etiology differs from that of type 1 diabetes, immune-cell infiltration and decrease in functional β-cell mass are also observed in type 2 diabetes (3).

Proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and γ-interferon (IFN-γ), produced by infiltrating leukocytes and by islet cells, play a central role in β-cell failure and in the development of diabetes (1,3–5). Prolonged exposure to cytokines leads to decreased capacity of β-cells to produce and release insulin in response to secretagogues and, in the long term, to destruction of the cells by apoptosis or necrosis. Thus, the elucidation of the molecular events occurring during immune-mediated injury is essential to determine the causes of diabetes and develop new treatments for the disease.

Cytokines induce modifications in gene expression through the activation of different transcription factors (1,3) that lead to upregulation of proteins exerting harmful actions on β-cells (6,7). In addition to transcription factors, other regulatory molecules make an important contribution to the control of gene expression (8). Eukaryotic cells contain hundreds of noncoding RNAs called microRNAs (miRNAs) that associate with the 3′ untranslated region of mRNAs (9), potentially inhibiting messenger translation of thousands of genes (10,11). Although we are only beginning to appreciate the potential of miRNAs as controllers of gene networks, there is already evidence that these molecules play a central role in many physiological processes and human diseases (12). miRNAs are also important regulators of specialized β-cell functions (13–16). Indeed, expression of appropriate levels of miR-375, miR-9, and miR-124a are required for insulin biosynthesis and for optimal release of the hormone in response to secretagogues (13–16).

The aim of this study was to investigate the possible involvement of miRNAs in cytokine-mediated β-cell damage and in the development of type 1 diabetes. We found that proinflammatory cytokines induce the expression of a subset of miRNAs, which alters insulin secretion and promotes β-cell apoptosis.

RESEARCH DESIGN AND METHODS

Insulin-secreting cell line culture and pancreatic islet isolation. The insulin-secreting cell line MIN6B1 and INS-1E were cultured as described (17,18). Transfections of MIN6 and INS-1E cells were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 60 pmol oligonucleotides.

Human pancreatic islets were provided by the Cell Isolation and Transplantation Center (University of Geneva) thanks to the ECIT "Islets for Research" distribution program sponsored by the Juvenile Diabetes Research Foundation and were cultured for 4 days in CMRL medium. Female NOD mice

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were obtained from The Jackson Laboratories (Bar Harbor, ME). Mouse pancreatic islets were isolated by collagenase digestion (19). After isolation, the islets were immediately processed for RNA purification and histological analysis.

Evaluation of islet lymphocytes. Islets isolated from NOD mice were fixed and sectioned at 1 μm thickness as previously described (20). The number of β-cells and lymphocytes was scored on 5–17 sections taken at 30-μm intervals. A minimum of 10 lymphocytes per islet was considered the threshold for the presence of peri- (lymphocytes around the islet) or intrainsulitis (lymphocytes within the islet). Given that the islets with such alterations were not distributed in a Gaussian way, values were expressed as medians and compared with the median test as provided by the Statistical Package for Social Sciences (SPSS, Chicago, IL). β-cell numbers were expressed as means ± SEM and compared with the ANOVA test, provided by the same software.

miRNA profiling. Total RNA from MIN6 cells treated for 24 h with either 10 ng/ml IL-1β or 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ was extracted using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX). Global miRNA profiling was performed by the LC Sciences microarray service (Houston, TX). The service included the analysis of the level of all mouse miRNAs available on the miRBase, version 9.2. Expression analysis was performed on a microarray platform using the μParaflo microfluidic chip technology (21).

miRNA detection, overexpression, and downregulation. RNA extraction for miRNA analysis was performed with the *mir*Vana isolation kit. Mature miRNA measurements were performed using a quantitative RT-PCR (qRT-PCR) miRNA Detection kit (Ambion). The results were normalized using cDNAs amplified with U6 primers in the same samples. To increase miRNA levels, the cells were transfected with RNA duplexes (Eurogentec, Seraing, Belgium) corresponding with the mature sequence of the noncoding RNA. A siRNA duplex directed against green fluorescent protein was used as a control. Endogenous activity of miRNAs was blocked by transfecting the corresponding clear-MiR miRNA inhibitor (Eurogentec, Seraing, Belgium).

Analysis of the expression of protein-coding genes. RNA extraction was performed with the RNAqueous isolation kit (Ambion). Conventional qRT-PCR was carried out as previously described (22).

Luciferase assays. The luciferase reporter plasmid driven by the miR-146a promoter and the corresponding construct in which the nuclear factor-κB (NF-κB) binding sites are mutated (23) were provided by Dr. D. Baltimore (California Institute of Technology, Pasadena, CA). The luciferase reporter plasmid driven by a 600-bp fragment of the rat insulin 2 promoter has previously been described (24). Luciferase activity was measured with a dual-luciferase reporter assay (Promega, Madison, WI) 2 days after transfection. Firefly luciferase activity was normalized for transfection efficiency with the SV40-driven Renilla activity generated by the psiCHECK-1 vector (Promega).

Secretion assay. MIN6 cells were transfected with RNA duplexes or antisense oligonucleotides. After 48 h, the cells were preincubated for 30 min in KREBS buffer containing 2 mmol/l glucose (25). The medium was then discarded, and the cells were incubated for 45 min in the same buffer (basal condition) or in a KREBS buffer containing 20 mmol/l glucose (stimulatory condition). The amount of insulin in the samples was assessed by ELISA (SPI-bio, Montigny-le-Bretonneux, France).

Western blots. Protein extracts were separated on acrylamide gels and transferred on polyvinylidine fluoride membranes, and immunoreactive bands were visualized by chemiluminescence (Amersham Biosciences). Antibodies against vesicle-associated membrane protein (VAMP)2 and Rab3a were purchased from Synaptic Systems (Goettingen, Germany). The antibody against syntaxin1a was bought from Sigma-Aldrich (St. Louis, MO) and that against actin from Chemicon International (Temecula, CA). Antibodies against c-Jun and *Bcl*II were from Cell Signaling (Danvers, MA) and those against phosphorylated c-Jun (Ser73) and TNF receptor–associated factor (TRAF)6 from Upstate Biotechnology (Temencula, CA). The antibody against IL-1 receptor–associated kinase (IRAK)1 was bought from Abcam (Cambridge, U.K.).

Cell death. We scored the cells displaying pycnotic nuclei upon Hoechst 33342 staining (25). The experiment was carried out blindly, and at least 500 cells per condition were analyzed. Alternatively, apoptosis was assessed by enzyme-linked immunoassay using a Cell Death Detection kit (Roche, Rotkreuz, Switzerland).

Statistical analysis. Statistical differences were tested by ANOVA. The experiments including more than two groups were first analyzed by ANOVA, and multiple comparisons of the means were then carried out using the post hoc Dunnett's test, with a discriminating P value of 0.05 (SAS statistical package; SAS, Carry, NC).

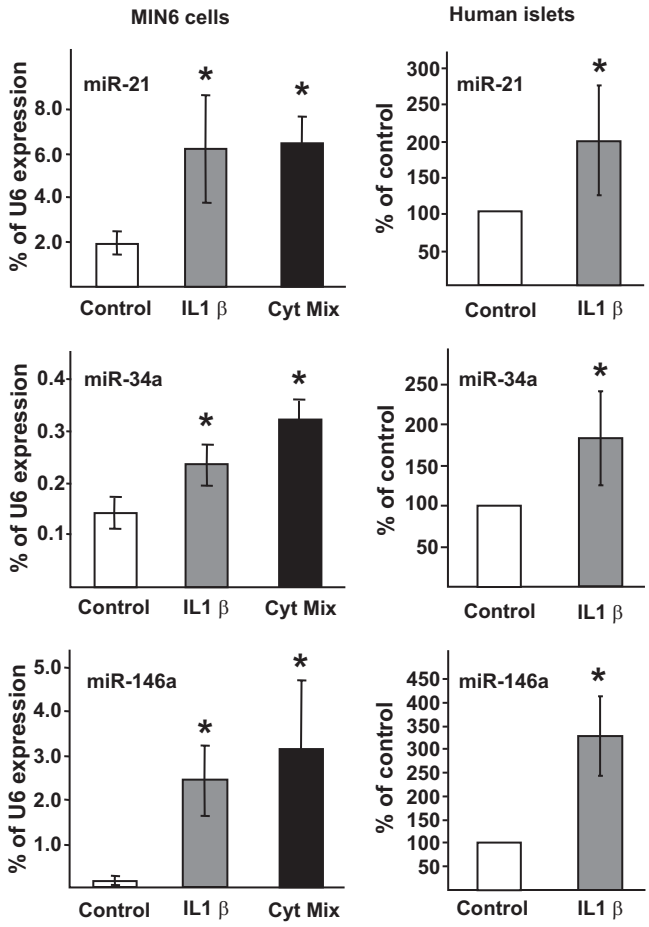

FIG. 1. Effect of IL-1β and of a mix of cytokines on the expression of miR-21, miR-34a, and miR-146a. *Left panel*: MIN6 cells were incubated for 24 h with 10 ng/ml IL-1β or with a mix of 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ. The expression of the indicated miRNAs was assessed by quantitative RT-PCR. The results are expressed as percent of the level of U6, which was measured in parallel in the same samples. They are the means ± SEM of three independent experiments. *Conditions that are significantly different from controls ($P < 0.05$). *Right panel*: Isolated human pancreatic islets were incubated for 24 h in the presence or absence of 10 ng/ml IL-1β. The expression of the indicated miRNAs was assessed by quantitative RT-PCR and is expressed as percent of the level of the corresponding miRNA in control islets. Data are shown as means ± SEM of four independent experiments. *Conditions significantly different from controls ($P < 0.05$; $n = 4$). Cyto, cytokine.

RESULTS

To investigate the possible involvement of miRNAs in cytokine-induced β-cell failure, the mouse insulin-secreting cell line MIN6 was incubated for 24 h in the presence of 10 ng/ml IL-1β or of a mix of 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ. Global miRNA expression profile was then determined by microarray analyses. The majority of the miRNAs was not affected by cytokine treatment (supplementary Table 1, available in an online appendix [http://diabetes.diabetesjournals.org/cgi/content/full/db09-0881/DC1]). However, miR-21, miR-34a, and miR-146a/b were significantly increased both by IL-1β and the cytokine mix. These findings were confirmed by qRT-PCR of MIN6 cells and isolated human pancreatic islets (Fig. 1), and similar results were also obtained with the rat insulin-secreting cell line INS-1E (supplementary Fig. 1). In one of the samples used for microarray analysis, we observed changes in miR-96 expression. However, the levels of miR-96 measured by qRT-PCR in four other independent

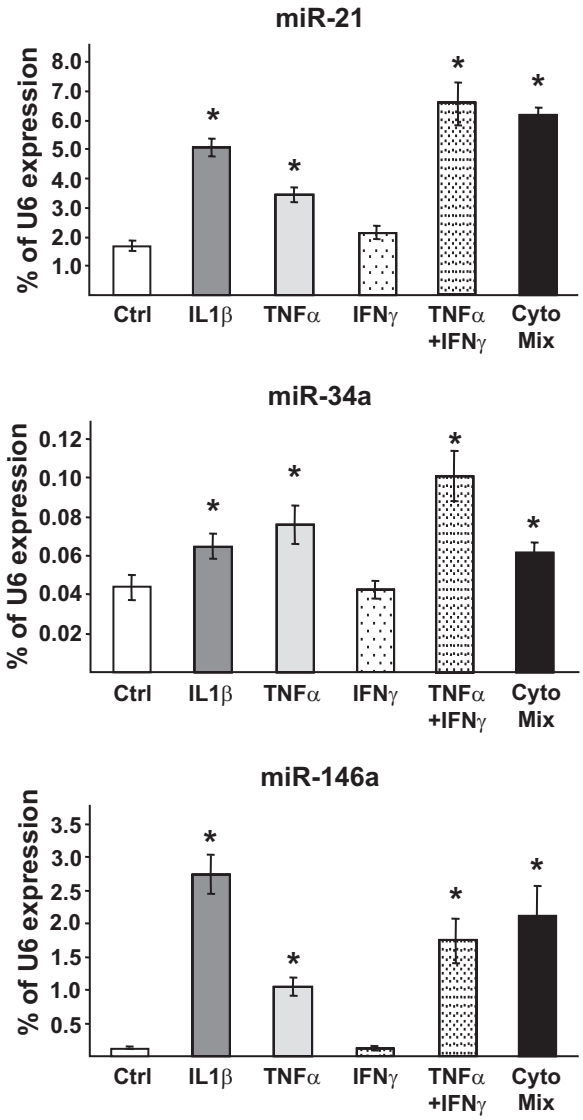

FIG. 2. Effect of different cytokine combinations on the expression of miR-21, miR-34a, and miR-146a. MIN6 cells were incubated in the presence of the indicated cytokines (10 ng/ml) for 24 h. The expression of miR-21 (*A*), miR-34a (*B*), and miR-146a (*C*) was assessed by quantitative RT-PCR. The results are expressed as percent of the level of U6 that was measured in parallel in the same samples. Data are shown as means ± SEM of three independent experiments. *Conditions that are significantly different from controls ($P < 0.05$). Cyto, cytokine.

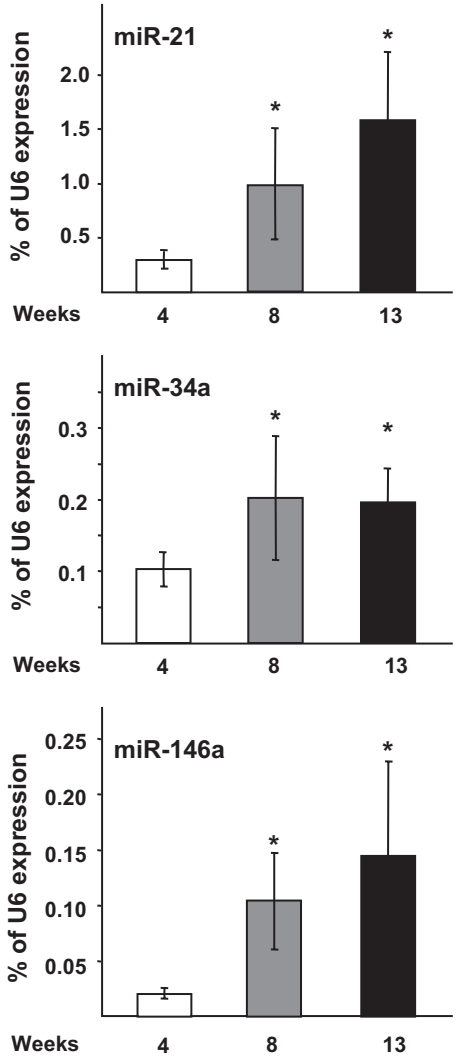

FIG. 3. Expression of miR-21, miR-34a, and miR-146a in pancreatic islets of NOD mice. Pancreatic islets were isolated from female NOD mice of different ages. Only animals displaying blood glucose levels within the normal range were included in the study. Blood glucose levels for the mice included in the study were as follows: 99 ± 5 mg/dl for 4-week-old mice ($n = 4$), 100 ± 8 mg/dl for 8-week-old mice ($n = 4$), and 132 ± 25 mg/dl for 13-week-old mice ($n = 9$). The levels of miR-21, miR-34a, and miR-146a were measured by quantitative RT-PCR and are given as % of U6 expression. Data are shown as means ± SEM of four independent experiments. *Conditions that are significantly different from controls ($P < 0.05$).

experiments represented 102 ± 11 and 104 ± 17% of those of controls in MIN6 cells treated with IL-1β and cytokine mix, respectively. Therefore, the role of this miRNA was not further investigated.

The induction of miR-146a (primers are not available for the selective detection of miR-146b by qRT-PCR) was detectable already after 6 h incubation with IL-1β, reached its maximum after 24 h, and remained stable until 72 h (supplementary Fig. 2). miR-21 and miR-34a expression was significantly higher than control cells only after 24 h incubation, and these miRNAs were not further increased after 48 or 72 h. Half-maximal induction of the three miRNAs was observed at ~0.1 ng/ml IL-1β, while maximal effect was achieved with 1 ng/ml (supplementary Fig. 3). Incubation of MIN6 cells with different combinations of cytokines revealed that IL-1β was, alone, the most potent inducer of miR-21 and miR-146a expression (Fig. 2). The level of these miRNAs was also increased by TNF-α but not by IFN-γ. The expression of miR-34a was stimulated to approximately the same extent by IL-1β and TNF-α, whereas IFN-γ again had no effect (Fig. 2).

We then tested whether the development of diabetes in NOD mice, a well-established type 1 diabetes model (26), is preceded by changes in the level of miRNAs in pancreatic islets. In NOD mice, peri-insulitis begins around a few islets 6–8 weeks after birth and characterizes a majority of islets at the age of ~13–14 weeks (27). At that time, the β-cell mass begins to decrease, resulting in elevation of blood glucose and development of diabetes. We measured miRNA expression in islets from female NOD mice at the ages of 4, 8, and 13 weeks displaying blood glucose levels within the normal range (Fig. 3). In islets from 8- and 13-week-old animals, the levels of miR-21 and miR-146a were significantly higher compared with those from

4-week-old mice (Fig. 3). miR-34a was also increased to a lesser extent in 8- and 13-week-old mice. In contrast, the level of miR-7, a miRNA selectively expressed in pancreatic islet cells (28), was not significantly altered (not shown). Evaluation of sections of islets, isolated like those extracted for quantitive PCR, showed that islets from 4-week-old animals contain very few lymphocytes. A majority of the islets isolated from 8-week-old NOD mice had small-to-moderate peri-insulitis, with a lower proportion of the islets also containing sufficient lymphocyte numbers to be scored for intra-islet insulitis (supplementary Fig. 4). The same phenotypes were seen in the islets isolated from normoglycemic, 14-week-old NOD mice in which the proportion of islets with intraislet insulitis and the numbers of lymphocytes per islet tended to increase, whereas β-cells tended to decrease (supplementary Fig. 4). However, a sizable proportion of islets of both 8- and 14-week-old animals did not display signs of insulitis, and those that did still featured relatively few intraislet lymphocytes (supplementary Fig. 4). These data document that the islets we isolated closely reflected the distribution of islets examined within the intact pancreas of NOD mice (data not shown).

In other cell systems, miR-146a is induced by the activation of the NF-κB pathway (23). Exposure of MIN6 cells to cytokines led to an increase in the expression of a luciferase reporter construct driven by the miR-146a promoter (Fig. 4). Mutation of the NF-κB binding sites in the miR-146a promoter (23) abolished this effect (Fig. 4), indicating that the cytokine-mediated induction of miR-146a in insulin-secreting cells results from NF-κB activation. Basal promoter activity was also reduced by mutation of the putative NF-κB binding sites (Fig. 4), suggesting a contribution of this transcription factor to the expression of miR-146a under resting conditions as well.

Next, we examined whether changes in the level of the three miRNAs induced by cytokines altered specific β-cell functions. Overexpression of miRNAs was achieved by transfection of RNA duplexes corresponding to the mature form of the miRNAs. Reduction of miRNA activities was obtained by transfecting antisense O-methyl RNA oligonucleotides (29). To verify the efficacy of this approach, the miRNAs and the anti-miRs were cotransfected with luciferase constructs containing specific miRNA binding sites in their 3′ untranslated region (UTR) (miRNA sensors). Overexpression of each miRNA led to a strong reduction in the luciferase activity of the corresponding sensor, whereas this activity was increased upon blockade of endogenous miRNA function by anti-miRs (supplementary Fig. 5).

We first assessed the impact of the three miRNAs on insulin gene expression. Overexpression of miR-21 or miR-146a did not significantly affect insulin content (Fig. 5*A*), insulin promoter activity (Fig. 5*C*), or proinsulin mRNA levels (not shown). In contrast, miR-34a overexpression led to a small decrease in insulin content (Fig. 5*A*) and insulin promoter activity (Fig. 5*C*), accompanied by a reduction in proinsulin mRNA level (Fig. 5*B*). As expected, incubation of MIN6 cells with IL-1β led to a dose-dependent impairment in the activity of the insulin promoter (Fig. 5*D*) and to a decrease in proinsulin mRNA content (Fig. 5*E*). Treatment of the cells with anti–miR-34a did not prevent the decrease in the activity of the insulin promoter (Fig. 5*D*) or proinsulin mRNA levels (Fig. 5*E*), suggesting that miR-34a does not contribute to these effects of the cytokine. Anti–miR-21 and anti–miR-146a were also unable to restore insulin biosynthesis in IL-1β–treated cells (not shown).

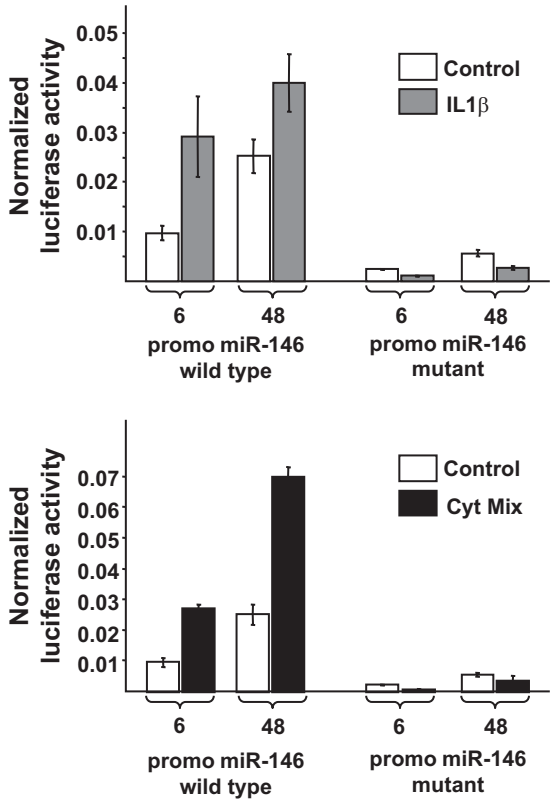

FIG. 4. The expression of miR-146a is controlled by the NF-κB pathway. *Upper panel*: MIN6 cells were transiently cotransfected with a plasmid leading to constitutive expression of Renilla luciferase and with Firefly luciferase reporter constructs driven either by the wild-type miR-146a promoter (promo miR-146 wt) or by a promoter lacking the putative NF-κB binding sites (promo miR-146 mutant). The cells were then treated either for 6 or 48 h with 10 ng/ml IL-1β. The figure shows a representative experiment out of five and presents the ratio between the Firefly and Renilla luciferase activities measured at the end of the IL-1β treatment. *Lower panel*: The same experiment as in the *upper panel*, except that the cells were treated with a mixture of cytokines (10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ). Cyt, cytokine.

We then tested whether the level of the three miRNAs can influence the secretory capacity of the cells. Overexpression of miR-34a and miR-21 did not affect basal secretion but reduced maximal, glucose-induced insulin release (Fig. 6*A*). miR-146a overexpression had no significant effect on insulin secretion (Fig. 6*A*). These observations cannot be merely attributed to changes in the number of cells/well. Indeed, the total amount of proteins/well was not significantly different between experimental conditions (data not shown). The defect in secretion observed in the presence of elevated levels of miR-21 and miR-34a was accompanied by a drop in the expression of VAMP2, a sensitive factor attachment protein receptor (SNARE) protein that is essential for β-cell exocytosis (30–32), and of the GTPase Rab3a (33) (Fig. 6*B*). Incubation of MIN6 cells for 24 h in the presence of 1 ng/ml IL-1β, a condition that alone does not significantly affect cell survival (data not shown), resulted in impaired glucose-induced insulin secretion (Fig. 6*C*). Defective insulin secretion was accompanied by a reduction in the expression of VAMP2 and Rab3a (Fig. 6*B*). Pretreatment of the cells exposed to 1 ng/ml IL-1β with anti–miR-21 or anti–miR-34a prevented the decrease of VAMP2 but not of Rab3a (Fig. 6*B*) and improved glucose-induced secretion in cytokine-

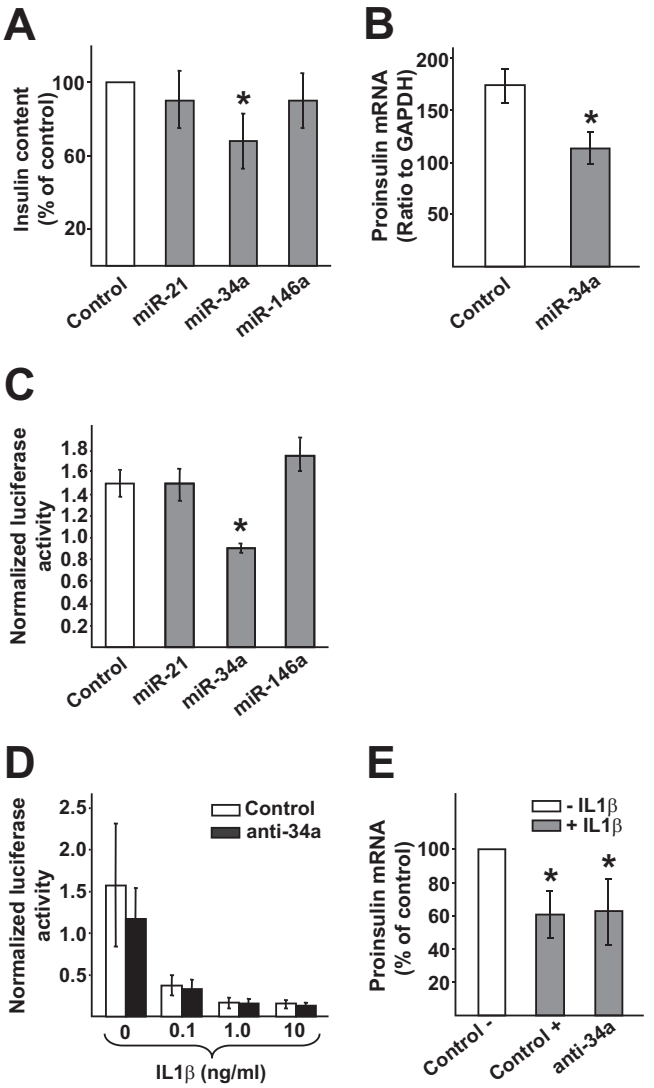

FIG. 5. Effect of miR-21, miR-34a, and miR-146a on insulin-promoter activity and insulin biosynthesis. *A*: MIN6 cells were transfected with a control RNA duplex (open bars) or with duplexes containing the mature forms of miR-21, miR-34a, or miR-146a (gray bars). Insulin content was measured 2 days later by ELISA. The results are expressed as percent of the values in control cells. *B*: Proinsulin and glyceraldehyde-3-phosphate dehydrogenase (GAPHD) mRNA levels in control MIN6 cells and in cells overexpressing miR-34a were assessed by quantitative RT-PCR. The results are expressed as the ratios between proinsulin and glyceraldehyde-3-phosphate dehydrogenase mRNA. *C*: MIN6 cells were transiently cotransfected with a Firefly luciferase reporter construct driven by the rat insulin promoter, a plasmid leading to constitutive expression of Renilla luciferase and RNA duplexes containing the indicated mature miRNAs. Luciferase activities were measured 2 days later. The results are expressed as the ratios between Firefly and Renilla luciferases. *D*: MIN6 cells were transiently cotransfected with a Firefly luciferase reporter construct driven by the rat insulin promoter, a plasmid leading to constitutive expression of Renilla luciferase and with a control antisense oligonucleotide (white bars) or with anti-34a (black bars). The day after, the cells were incubated for 24 h in the presence of the indicated concentrations of IL-1β. The results are expressed as the ratios between Firefly and Renilla luciferases. *E*: MIN6 cells were transfected with a control oligonucleotide or with anti-34a. The day after, the cells were incubated for 24 h in the presence (gray bars) or absence (white bars) of 10 ng/ml IL-1β. Proinsulin mRNA levels were determined by quantitative RT-PCR. The results are expressed as percent of the level of proinsulin mRNA in untreated control cells. All data are shown as means ± SEM of three (*B* and *E*), four (*C* and *D*), or eight (*A*) independent experiments. *Conditions that are significantly different from controls ($P < 0.05$).

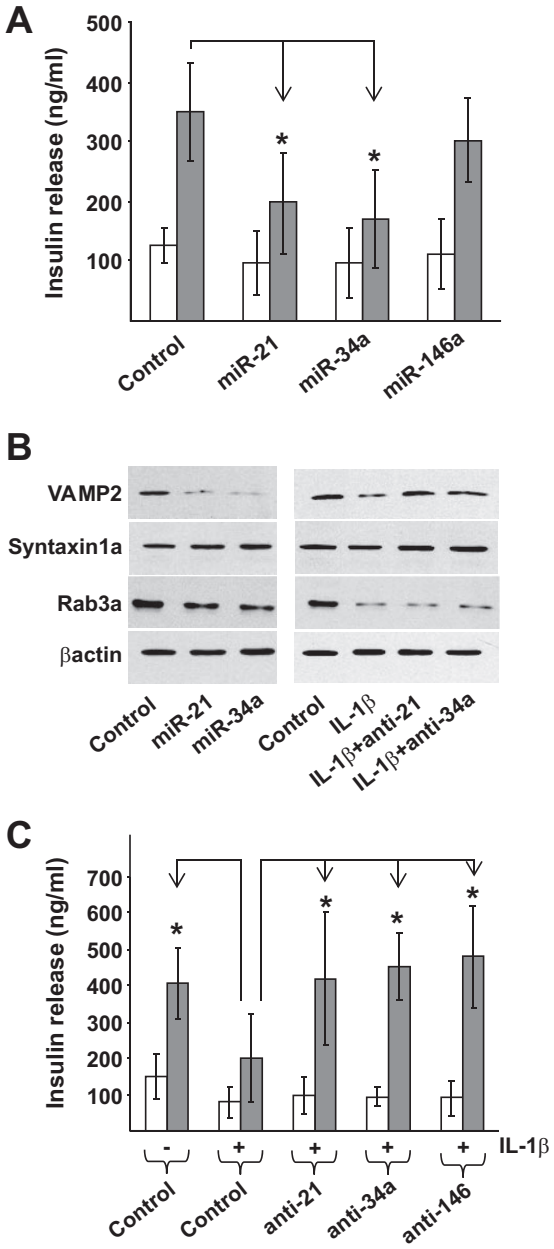

FIG. 6. Involvement of miR-21, miR-34a, and miR-146a in insulin secretion. *A*: MIN6 cells were transiently transfected with RNA duplexes containing the mature sequence of the indicated miRNAs. Control cells were transfected with a siRNA duplex against green fluorescent protein. Two days later, the cells were preincubated for 30 min in Krebs-Ringer buffer containing 2 mmol/l glucose and successively incubated either in the same buffer (white bars) or in Krebs-Ringer buffer containing 20 mmol/l glucose (gray bars). The amount of insulin released during the incubation period was measured by ELISA. The results are the means ± SEM of three independent experiments. *Significantly different ($P < 0.05$) from control. *B*: MIN6 cells were transiently transfected with RNA duplexes leading to the overexpression of miR-21 or miR-34a. The expression of the indicated components of the machinery governing insulin exocytosis was analyzed by Western blotting (*left panels*). MIN6 cells were transiently transfected with a control oligonucleotide (control) or with O-methyl antisense oligonucleotides blocking the activity of miR-21 or miR-34a (anti-21 and anti-34a, respectively). The day after, the cells were incubated with or without IL-1β for 24 h. The expression of the indicated proteins was analyzed by Western blotting (*right panel*). The figure shows the results of a representative experiment out of three. *C*: MIN6 cells were transfected with the indicated anti-miRs. The day after, they were incubated for 24 h with (+) or without (−) IL-1β (1 ng/ml). Insulin release in the presence of 2 and 20 mmol/l glucose was assessed by ELISA. The results are the means ± SEM of five independent experiments. *High glucose conditions that are significantly different from control cells incubated in the presence of IL-1β ($P < 0.05$).

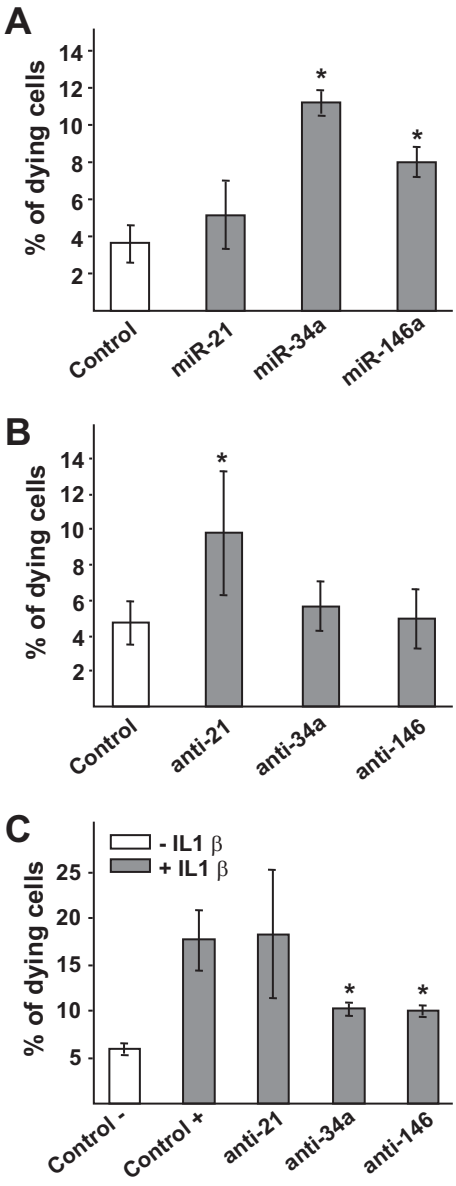

FIG. 7. Effect of miR-21, miR-34a, and miR-146a on apoptosis. *A*: MIN6 cells were transfected with a control RNA duplex (open bars) or with RNA duplexes, leading to a rise of the indicated miRNAs (gray bars). The fraction of dying cells was determined 3 days later by scoring the cells displaying picnotic nuclei. *B*: MIN6 cells were transfected with a control oligonucleotide (open bars) or with O-methyl antisense oligonucleotides blocking the activity of miR-21, miR-34a, or miR-146a (gray bars). Apoptotic cells were scored 3 days later as described above. *C*: MIN6 cells transfected with a control oligonucleotide (open bars) or with anti-miRs (gray bars). Two days later, the cells were incubated for 24 h with (gray bars) or without (white bars) 10 ng/ml IL-1β. Dying cells were scored as described above. Data are shown as means ± SEM of six (*A*) or four (*B*) and independent (*C*) experiments. *Conditions that are significantly different from controls ($P < 0.05$).

treated cells (Fig. 6*C*). Interestingly, although alone overexpression of miR-146a did not affect glucose-induced insulin release (Fig. 6*A*), anti–miR-146 pretreatment was able to alleviate the secretory defect elicited by IL-1β (Fig. 6*C*).

Prolonged exposure of β-cells to cytokines sensitizes them to apoptosis (1,3,5). Overexpression of miR-34a or miR-146a increased the number of cells undergoing apoptosis, whereas overexpression of miR-21 had no significant effect (Fig. 7*A*). In agreement with previously published data (34), anti–miR-21 treatment led to an increase in apoptosis (Fig. 7*B*). In contrast, reducing miR-34a or miR-146a levels did not affect cell survival. Treatment of the cells with anti–miR-34a or anti–miR-146 diminished the number of dying cells in the presence of high doses of IL-1β (10 ng/ml) (Fig. 7*C*) or the cytokine mix (data not shown). Similar results were obtained when apoptosis was assessed specifically using an alternative method (supplementary Fig. 6).

We have previously shown that the protective effect of anti–miR-34a on apoptosis may be due to the capacity of miR-34a to control the expression of the antiapoptotic protein BclII (25). Indeed, overexpression of miR-34a led to a decrease in BclII and anti–miR-34a raised the level of the antiapoptotic protein in the presence of IL-1β (supplementary Fig. 7), potentially explaining part of the protective effect of the anti-miR. The mode of action of miR-146a, the miRNA displaying the most dramatic expression changes in response to cytokines, is presently unknown. In other cell systems, miR-146a controls the expression of IRAK1 and TRAF6. Overexpression of miR-146a in MIN6 cells caused a decrease in IRAK1 and TRAF6, whereas blockade of miR-146a raised the level of these two components of IL-1β signaling (supplementary Fig. 8). Although these findings confirm the capacity of miR-146a to modulate IL-1β signaling, they are unlikely to account for the protective effects of anti–miR-146. However, we discovered that the induction of c-Jun expression elicited by IL-1β is reduced in cells lacking miR-146a. In these cells, the sixfold increase in c-Jun mRNA observed upon 1 h incubation with IL-1β was reduced by 38 ± 4% ($n = 3$) (data not shown). Moreover, the total amount of c-Jun and the phosphorylated fraction of the transcription factor present in IL-1β-treated cells were reduced by >60% (Fig. 8).

DISCUSSION

During development of type 1 diabetes, pancreatic β-cells are exposed to proinflammatory cytokines produced by invading leukocytes and by the islet cells themselves. Chronic exposure to these inflammatory mediators results in changes in gene expression culminating in the loss of β-cell functions and apoptosis (6,8,35). miRNAs are emerging as important regulators of gene expression, but their involvement in cytokine-mediated β-cell failure has not been evaluated. In this study, we identified three miRNAs that are induced in cytokine-treated β-cells and in pre-diabetic NOD mice. The increase in miRNAs observed in NOD mice between 4 and 8 weeks of age cannot be attributed to immune cells carried out during the isolation procedure, given that at the latter time point, these cells were still quite rare in most islets. In the islets isolated from normoglycemic 14-week-old NOD mice, an increasing number of lymphocytes surrounded the islets showing insulitis, but these cells were still outnumbered by β-cells and did not express detectable levels of the three miRNAs, as evaluated by in situ hybridization (data not shown). Moreover, the level of miR-21 and miR-34a is higher in islets than immune tissues, and that of miR-146a is only two to three times lower (supplementary Fig. 9). Although we cannot exclude that the immune cells surrounding the islets express miRNA levels different from those in spleen and thymus, these findings make it very unlikely that lymphocyte infiltration is the sole determinant of the changes in miRNA that we observed. Rather, the finding that the same miRNAs are modulated by cytokines in insulin-producing cell lines and islets isolated from nondiabetic donors strongly suggests that the miRNA changes occurred in islet cells.

Interestingly, prolonged exposure of β-cells to palmitate

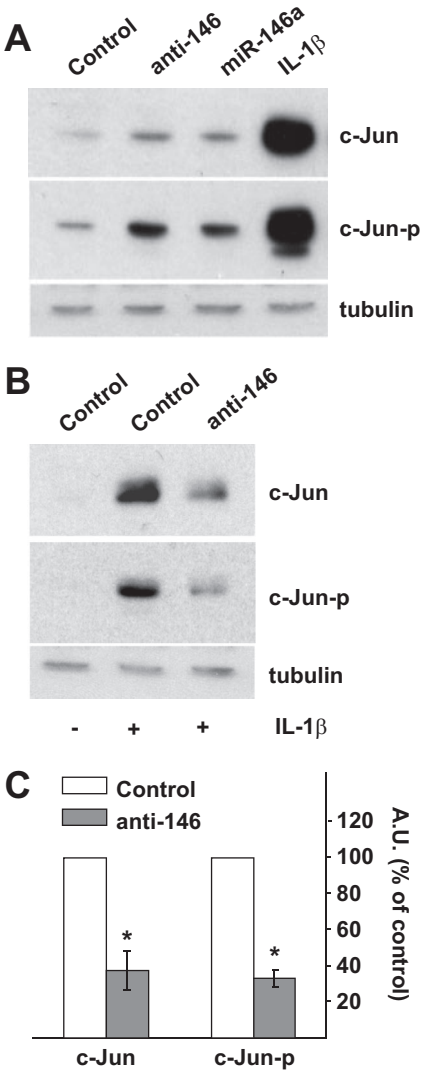

FIG. 8. Impact of miR-146a levels on c-Jun expression. *A*: MIN6 cells were transfected with either control oligonucleotides or oligonucleotides leading to silencing (anti-146a) or overexpression (miR-146a) of miR-146a. Two days later, some of the cells were treated with 10 ng/ml IL-1β for 1 h. The total amount of c-Jun present in the cells at the end of the treatment (c-Jun) and the fraction of the transcription factor in phosphorylated form (c-Jun-p) were assessed by Western blotting. Equal loading in each lane was verified using an antibody against tubulin. *B*: MIN6 cells were transfected with either control oligonucleotides or with anti-146a. Two days later, the cells were incubated in the presence (+) or absence (−) of 10 ng/ml IL-1β for 1 h. Total c-Jun and phosphorylated c-Jun were detected as above. In this case, the exposure of the film was reduced to prevent its saturation in the lanes containing the samples treated with IL-1β. The figure shows a representative experiment out of three. *C*: Quantification of the expression of c-Jun and c-Jun-p by densitometric scanning of the films. Data are shown as means ± SEM of three independent experiments. *Conditions that are significantly different from controls ($P < 0.05$).

and proinflammatory cytokines affects the expression of an overlapping group of miRNAs, and changes in miR-34a and miR-146a expression are observed in both type 1 (NOD mice [this study]) and type 2 (*db/db* mice [25]) diabetes models, suggesting analogies in the mechanisms leading to β-cell failure. In agreement with this hypothesis, it has been proposed that islet inflammation plays a role in both type 1 and type 2 diabetes (3).

The most striking changes in the miRNA profile concerned miR-146a. The expression of this miRNA is controlled by NF-κB, a transcription factor involved in cytokine-mediated β-cell dysfunction and in the development of diabetes. Indeed, overexpression of an NF-κB super-repressor protects β-cells against cytokine-induced apoptosis (36), and transgenic mice expressing the super-repressor are resistant to diabetes induced by multiple streptozotocin injections (37). Inhibition of inducible nitric oxide synthase with 1 mmol/l *N*-ω-nitro-L-arginine methyl ester did not prevent the induction of miR-146a, confirming that the miRNA is directly controlled by NF-κB (E.R., A.B., S.G., N.L.-M., A.A., P.M., R.R., unpublished observations). Blockade of miR-146a improved glucose-stimulated insulin secretion in IL-1β–treated cells and partially protected the cells against apoptosis triggered by cytokines. These findings indicate that part of the effects of the activation of the NF-κB pathway in β-cells may be mediated by the induction of miR-146a. Although upregulation and downregulation of the levels of miR-146a elicited opposite effects on β-cell death, overexpression of miR-146a per se was ineffective on insulin secretion. One possible explanation for this finding is that, under resting conditions, the transfected miR-146a cannot access all its potential mRNA targets because the binding sites are masked by RNA-binding proteins (38,39). Cytokines are known to affect expression and function of several RNA-binding proteins (8). Thus, a subset of targets may become accessible and repressed only upon IL-1β treatment, unveiling the role played by miR-146a in the secretory function of β-cells. Another possibility is that the anti-miR treatment allows for the expression of genes already fully repressed by miR-146a and that, therefore, cannot be further reduced by the overexpression of the miRNA.

While sustained activation of NF-κB has a deleterious impact on β-cells, transient and moderate activation of this signaling cascade is required for proper glucose-stimulated insulin secretion and β-cell survival (40,41). miR-146a is expected to have different affinities for its numerous targets. Interestingly, TRAF6 and IRAK1, two key components of the IL-1β and TNF-α signaling cascade, are among the best-predicted targets of miR-146a (10,11), and repression was experimentally confirmed in this study. A moderate induction of miR-146a may contribute to modulation of the response of the cells to these two cytokines. In contrast, a strong and prolonged induction of miR-146a in the presence of cytokines may result in the repression of additional targets with a deleterious impact on glucose-induced insulin secretion and β-cell survival. The mechanisms behind the contribution of miR-146a to the adverse effects of cytokines on β-cells remain to be fully elucidated. We found that silencing of miR-146a attenuates c-Jun induction in IL-1β–treated cells without affecting c-Jun NH_2-terminal kinase activity, which possibly explains some of the protective effects of anti–miR-146 (42). This effect could either be caused by modifications in the level of components controlling the transcriptional activity of the c-Jun promoter or by changes in the expression of RNA-binding proteins that govern c-Jun mRNA stability (43). Each miRNA is known to generate subtle modifications in the level of hundreds of targets (44,45). The attenuation of c-Jun induction is likely to result from the synergistic effect of these changes and probably not from repression of a single target mRNA. Thus, future studies should aim at analyzing the global impact of miR-146a on the proteome and, in particular, on the expression of key proteins involved in glucose metabolism, stimulus-secretion coupling, and apoptosis rather than attempting to identify individual targets.

miR-21 is strongly upregulated in cytokine-treated β-cells and in NOD mice during islet inflammation. In myeloma cells, the expression of miR-21 is induced by IL-6 through a signaling cascade involving the transcription factor Stat3 (46). Whether a similar mechanism is operating in β-cells exposed to cytokines remains to be established. Overexpression of miR-21 in MIN6 cells led to impairment in insulin secretion stimulated by glucose. A decrease in the expression of VAMP2, a secretory granule protein that is essential for insulin exocytosis (30–32), may contribute to the defect in hormone release in miR-21–overexpressing cells. miR-21 adds up to the growing list of miRNAs capable of modulating the expression of components of the machinery of exocytosis of pancreatic β-cells (14,15). The 3′UTR of VAMP2 mRNA does not contain miR-21 recognition sites (10,11), which suggests an indirect effect of the miRNA. Blockade of miR-21 restored VAMP2 expression in IL-1β–treated cells and alleviated the impairment in glucose-induced secretion in IL-1β–treated cells, indicating that the induction of this miRNA can contribute to the effect of the cytokine. Changes in the expression of VAMP2 are unlikely to explain all the effects of miR-21, and possible modifications in the level of other key genes involved in glucose metabolism and stimulus-secretion coupling should be further explored.

Overexpression of miR-21 did not affect survival of MIN6 cells, but knockdown of the miRNA promoted apoptosis. This finding is in agreement with recent reports showing that miR-21 functions as an oncogene by translationally repressing the tumor suppressor gene programmed cell death 4 (47,48). Indeed, anti–miR-21 treatment of mammary carcinoma cells increased apoptosis and decreased cell proliferation (49). A similar mechanism may also operate in MIN6 cells transfected with anti–miR-21.

Among the miRNAs investigated, miR-34a was the one displaying less variation in cells exposed to cytokines. However, possibly because of a fine balance between the level of the miRNA and of its cellular targets, miR-34a significantly contributed to the cytotoxic effects of the cytokines. We recently demonstrated that in insulin-secreting cells, activation of the p53 pathway induces the expression of miR-34a (25). The increase of miR-34a elicited by different cytokines was accompanied by a rise of p53 expression (data not shown), suggesting that activation of this transcription factor may be involved in the induction of this miRNA in response to IL-1β and TNF-α. However, at present the contribution of other important signaling pathways triggered by these cytokines cannot be ruled out and will need to be investigated in detail in future studies.

Because in β-cells this miRNA can target important proteins such as VAMP2 and the antiapoptotic protein BclII (25), miR-34a could significantly contribute to cytokine-mediated β-cell dysfunction. Indeed, anti-miR34a treatment partially prevented the decrease in the expression of VAMP2 and BclII and ameliorated glucose-induced secretion and survival in IL-1β–treated cells. Overexpression of miR-34a reduced insulin promoter activity and diminished proinsulin mRNA levels and insulin content. However, the effect was relatively modest compared with that of IL-1β, and blockade of miR-34a did not restore insulin-promoter activity in cytokine-treated cells. This suggests only a marginal contribution, if any, of miR-34a to the deleterious impact of IL-1β on insulin biosynthesis.

We have shown that proinflammatory cytokines elicit modifications in miRNA expression that impact on β-cell activities. In this study, the functional improvement achieved by blocking these miRNAs has been evaluated in insulinoma cells. Attempts were made to perform similar studies in primary rat β-cells using a published siRNA transfection protocol (50,51). Unfortunately, using this approach, downregulation of miRNAs was insufficient (15–20%) (data not shown) for the investigatation of restoration of β-cell functions in cytokine-treated cells. Although the mechanisms leading to cytokine-mediated dysfunction and apoptosis in insulin-secreting cell lines are not fundamentally different from primary β-cells (52), the development of methodologies capable of efficiently modulating miRNA expression in primary β-cells in vitro and, possibly, in vivo are urgently needed to verify our findings and assess the contribution of miR-21, miR-34a, and miR-146a in the development of type 1 diabetes. Moreover, proteomic approaches that allow for large-scale assessment of protein expression (44,45) will be necessary for investigating in detail the molecular mechanisms underlying the effects of miR-21, miR-34a, and miR-146a. A global picture of the changes in protein expression occurring in cytokine-treated β-cells in the presence or absence of these three miRNAs should provide new insights into the causes of β-cell failure underlying the development of diabetes.

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