HDLs Protect Pancreatic β-Cells Against ER Stress by Restoring Protein Folding and Trafficking

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Endoplasmic reticulum (ER) homeostasis alteration contributes to pancreatic β-cell dysfunction and death and favors the development of diabetes. In this study, we demonstrate that HDLs protect β-cells against ER stress induced by thapsigargin, cyclopiazonic acid, palmitate, insulin overexpression, and high glucose concentrations. ER stress marker induction and ER morphology disruption mediated by these stimuli were inhibited by HDLs. Using a temperature-sensitive viral glycoprotein folding mutant, we show that HDLs correct impaired protein trafficking and folding induced by thapsigargin and palmitate. The ability of HDLs to protect β-cells against ER stress was inhibited by brefeldin A, an ER to Golgi trafficking blocker. These results indicate that HDLs restore ER homeostasis in response to ER stress, which is required for their ability to promote β-cell survival. This study identifies a cellular mechanism mediating the beneficial effect of HDLs on β-cells against ER stress-inducing factors. ***Diabetes*** **61:1100–1111, 2012**

Pancreatic β-cells have a highly developed endoplasmic reticulum (ER) that reflects their physiological function as insulin-secreting cells. There is ample evidence indicating that alterations in ER homeostasis in β-cells affect their physiological function, increase their susceptibility to apoptosis, and contribute to the development of diabetes (1,2). Conversely, several of the factors that are involved in β-cell failure, including free fatty acids (FFAs), high glucose concentrations, and sustained insulin secretion, are known to induce ER stress in these cells (2–5). In response to ER stress, the protein chaperone BiP (immunoglobulin heavy chain–binding protein) dissociates from the ER transmembrane proteins ATF6 (activating transcription factor 6), IRE1α (inositol requiring 1-α), and PERK (protein kinase RNA–like endoplasmic reticulum kinase), allowing BiP to bind to unfolded or misfolded proteins to assist in their (re)folding. Dissociation from BiP also leads to IRE1α and PERK stimulation, and ATF6 that is no longer bound to BiP translocates to the Golgi where it is cleaved and activated. The ensuing signaling events turn on unfolded protein response genes that encode, on one hand, proteins favoring the export and degradation of misfolded proteins and, on the other hand, protein chaperones, including BiP, to increase the folding capacity of the ER. However, if ER stress is too strong and sustained, the transcription factor CHOP (C/EBP homologous protein-10) is expressed, leading to apoptosis by decreasing the expression of the antiapoptotic Bcl-2 protein and by turning on the expression of apoptotic inducers such as death receptor 5 and Bim (6). In mice, genetic deletion of apoptotic mediators of the ER stress response (e.g., CHOP) can delay the development of diabetes (7). In humans, ER stress markers are associated with diabetes (8–10).

HDLs have crucial functions in cholesterol and lipid transport in the blood (11). In addition, HDLs exert multiple beneficial actions on cells by inducing antioxidative, anti-inflammatory, and antiapoptotic responses (12). Reduced levels of HDLs or HDL dysfunctions could therefore represent situations where the protective defense of an organism against metabolic stress is compromised. This is consistent with the fact that low HDL-cholesterol level is an independent risk factor for the development of type 2 diabetes (13,14). Reciprocally, most interventions that lead to increased HDL levels in humans are also known to reduce the risk of developing diabetes (15).

HDLs from diabetic patients display altered composition, notably higher triglyceride content, and reduced cholesterol esters (16), and they are also more oxidized than HDLs from control subjects (17). HDL modifications can alter their functionality. It has been shown, for example, that oxidized HDLs lose their ability to mediate cholesterol efflux (18).

The beneficial effect of HDLs against diabetes has been directly observed in humans where infusion of recombinant HDLs was found to improve β-cell function (19). This is again in line with the idea that HDLs have a positive effect on β-cell function and survival. Additionally, HDLs protect β-cells from cytokines and serum deprivation–induced apoptosis (20). Furthermore, HDLs block oxidized LDL-induced cell death (21,22) and reduce apoptosis induced by high glucose concentrations and ER stress inducers (23,24). HDLs have also been reported to favor insulin secretion in vitro (25). However, the mechanisms underlying the beneficial effects of HDLs on β-cells remain largely unknown.

Characterizing how HDLs protect β-cells from ER stress is important in the context of the known antidiabetogenic function of HDLs and their capacity to inhibit β-cell apoptosis. In this study, we provide evidence that HDLs protect β-cells against ER stress–inducing stimuli by improving protein folding and trafficking in the ER.

RESEARCH DESIGN AND METHODS

Western blot analysis, cell transfection, apoptosis determination, immunocytochemistry, RNA extraction, reverse transcription, quantitative PCR, lentivirus

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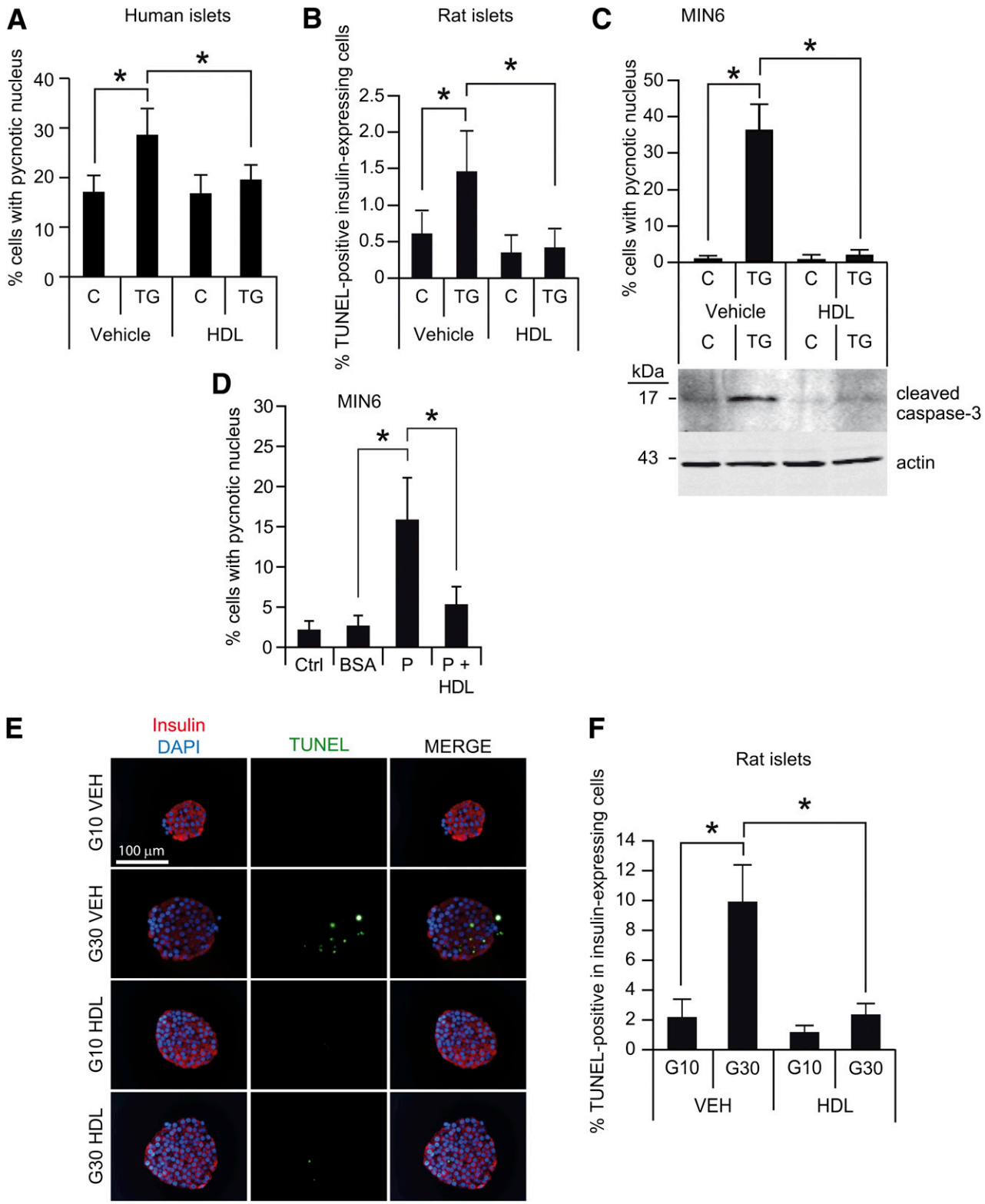

FIG. 1. HDLs protect β-cells against apoptosis induced by ER stress. *A*: Human islets from cadaveric donors were dissociated using trypsin and plated. The next day, islets were treated (TG) or not (control [C]) with 10 µmol/L TG in the presence (HDL) or in the absence (vehicle [VEH]) of HDLs for 24 h. Cells were then fixed, and apoptosis was assessed by scoring pycnotic nucleus. *B*: Cultured rat islets were incubated 24 h in serum-free RPMI 1640 medium containing 5 g/L BSA and 10 mmol/L glucose with the indicated combinations of 1 µmol/L TG and 1 mmol/L HDLs. Cell death was determined by transferase-mediated dUTP nick-end labeling (TUNEL) in insulin-expressing cells on histological sections of the islets. Results are expressed as the percentage of apoptotic cells among insulin-positive cells in a given islet section. A minimum of 2,000 cells from at least 20 islets have been scored from two independent experiments. *C*: MIN6 cells were treated with 0.5 µmol/L TG in the presence or in the absence of 1 mmol/L HDLs for 24 h. Cells were then fixed, and apoptosis was determined. Alternatively, the cells were lysed and the extent of caspase-3 activation was assessed by Western blotting using an antibody recognizing the cleaved active form of the protease. An actin-specific antibody was also used on the same blot to assess the evenness of loading. *D*: MIN6 cells were left untreated (control [Ctrl]) or treated for 48 h with 0.3% BSA (BSA) or 0.3% BSA/0.4 mmol/L palmitate (P) in the presence or in the absence of 1 mmol/L HDLs. Apoptosis was then scored as in

preparation, ^{35}S-methionine incorporation, and nuclear extract preparation were performed as described (23,26). Transferase-mediated dUTP nick-end labeling assay on rat islets was performed as described earlier (5).

Antibodies. Antibodies recognizing CHOP and BiP were from Santa Cruz (catalog No. 7351 and 13968, respectively). Antibodies specific for actin, caspase-3, phospho c-Jun N-terminal kinase (JNK), total JNK, and phospho-PERK were from Cell Signaling (catalog No. 4968, 9661, 3179, 9252, and 9251, respectively). 1E9 (called I14 in [27]) monoclonal antibody recognizing the correctly folded form of vesicular stomatitis virus glycoprotein (VSVG), 17-2-21-4 monoclonal antibody recognizing the VSVG protein exoplasmic domain and mouse IgG1-κ monoclonal antibody specific for GM130 (Golgi matrix protein of 130 kDa) (BD Biosciences, catalog No. 610822) were used for immunocytochemistry.

Primers. 18S sense (5′-GCAATTATTCCCCATGAACG-3′), antisense (5′-GG CCTCACTAAACCATCCAA-3′). CHOP sense (5′-TTCACTACTCTTGACCCT GCGTC-3′), antisense (5′-CACTGACCACTCTGTTTCCGTTTC-3′), spliced X-box binding protein 1 (XBP1) sense (5′-GAGTCCGCAGCAGGTG-3′), antisense (5′-GTGTCAGAGTCCATGGGA-3′), total XBP1 sense (5′-AAGAACACGCTTGG GAATGG-3′), antisense (5′-ACTCCCCTTGGCCTCCAC-3′).

Cells and cell culture. The MIN6B1 mouse insulinoma cell line (28) (referred here as MIN6) was cultured as described previously (23). Human islets were provided through the ECIT Islet for Basic Research program (JDRF award 31-2008-413). Islets were obtained from four different donors (see Supplementary Table 1 for the clinical characteristics of the donors). They were cultured in CMRL-1066 (GIBCO, catalog No. 21530) medium containing 5 mmol/L glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FCS. Islets were trypsinized with trypsin-EDTA 0.5× for 6 min with pipetting every minute and then plated at a density of 150,000 cells per well (24-well plates). The following day, islets were treated as indicated in the figures. Male Wistar rat islet isolation was performed as described earlier (5). All animal experimentations were approved by the local Institutional Committee on Animal Experimentation of the Faculty of Medicine of the Université catholique de Louvain (Project UCL/MD/2009/009).

Lipoprotein preparation and purification. Plasma lipoprotein fractions from human healthy donor serum were isolated on NaBr gradients by sequential ultracentrifugation, as described previously (29,30). The VLDL/IDL fraction (density 1.019) is isolated first, followed by the LDL fraction (density 1.063), and finally the HDL fraction (density 1.21). The fractions were dialyzed 48 h against PBS, 100 μmol/L EDTA, and stored at 4°C. Before use, HDLs were dialyzed twice 24 h against PBS. The medium used for dialysis (labeled "vehicle" in the figures) and HDLs were filtered through 0.22-μm filters. The cholesterol concentration of the fractions was measured by an enzymatic in vitro assay from Roche Applied Science (catalog No. 2016630). The HDL and vehicle fractions were used within a 14-day period. Experiments using HDLs were done with preparations from different donors or with mixed preparations and used at 1 mmol/L cholesterol.

XBP1 mRNA splicing. Touchdown PCRs were performed using the Taq polymerase from Promega. PCR products were loaded on a 4% agarose gel and run for about 6 h to discriminate the 26 nucleotides difference between the expected spliced and unspliced forms. For rat islets, XBP1 mRNA splicing was measured as described earlier (5). In Fig. 2*A* (*middle panel*) and in Fig. 4*B*, quantitative PCR was performed using primers described previously (31).

Plasmids. Plasmids mIns2-green fluorescent protein (GFP) (No. 688) and mIns2(C96Y)-GFP (No. 689), corresponding to pEGFP-Ins2-WT and pEGFP-Ins2-C96Y, were described in ref. 7 and were subcloned into the TRIP-PGK-ATGm-MCS-WHV* lentiviral vector (No. 349) generating plasmids mIns2-GFP.lti (No. 751) and mIns2(C96Y)-GFP.lti (No. 752). Plasmid pEGFP-VSVG (No. 729), obtained from Addgene, was subcloned into TRIP-PGK-ATGm-MCS-WHV* generating plasmid temperature-sensitive (ts)VSVG-GFP.lti (No. 730).

siRNA. siRNA, ordered from Microsynth (Balgach, Switzerland), sequences were as follows: siSRB1: 5′-AGG UCA ACA UCA CCU UCA ATT-3′ and small interfering GFP 5′-GAC GUA AAC GGC CAC AAG UUG-3′.

Folding assay. MIN6 cells were plated on coverslips and infected with 2.5 mL of a tsVSVG-GFP fusion protein-encoding lentivirus. Two days later, the cells were treated as mentioned in the figure legends. Cells were then fixed and cells on coverslips were subjected to immunocytochemistry labeling to detect correctly folded VSVG. At 32°C, the ts VSVG-GFP is synthesized on the ER and transits through the Golgi before reaching the plasma membrane. At the restrictive 40°C temperature, this protein is misfolded and accumulates in the ER. In neither case does the protein accumulate in the cytoplasm (32).

Electron microscopy. MIN6 cells were plated in poly-L-lysine (0.01%, Sigma-Aldrich, catalog No. P4832)–coated glass slides (LabTek Chamber Slides, catalog No. 177399) at a density of 110,000 cells per slide (area = 1.8 cm^2), cultured for 3 days, and finally treated as indicated in the figures. Cells were then fixed 2 h in 2.5% glutaraldehyde (Electron Microscopy Sciences, catalog No. 16220) dissolved in 0.1 mol/L phosphate buffer (PB), pH 7.4. After three washes in PB, MIN6 cells were postfixed for 1 h in 1% osmium tetroxide (Electron Microscopy Sciences, catalog No. 19150) in PB and then stained with ethanol 70% containing 1% uranyl acetate (Sigma-Aldrich, catalog No. 73943) for 20 min. MIN6 cells were dehydrated in graded alcohol series and embedded in epon (Electron Microscopy Sciences, catalog No. 13940). Ultrathin sections (with silver to gray interference) were cut with a diamond knife (Diatome), mounted on Formvar-coated single slot grids, and then counterstained with 3% uranyl acetate for 10 min and then with lead citrate (0.2%, Sigma-Aldrich, catalog No. 15326) for 10 min. Sections were visualized using a Philips CM100 transmission electron microscope.

Palmitate preparation. HEPES-buffered Krebs Ringer (KRBH) solution (120 mmol/L NaCl, 4 mmol/L KH_2PO_4, 20 mmol/L HEPES, 2 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 5 mmol/L $NaHCO_3$) was equilibrated in a cell culture incubator at 37°C, 5% CO_2 for 1 h. The pH was then adjusted to 7.4, and free fatty acid BSA (Sigma-Aldrich; catalog No. A60003) was dissolved to a 5% concentration. The solution was then filtrated. Palmitate sodium salt was finally dissolved in the KRBH-BSA solution to a 6.7 mmol/L concentration.

Oxidized HDL. HDLs were oxidized at 37°C using 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) as described previously (33). The reaction was stopped by placing the mixture on ice, and AAPH was removed by dialysis. Lipoperoxide concentrations were determined using the Fox assay (34).

Data presentation and statistics. Results are expressed as the mean ± 95% confidence intervals (CI) of three independent experiments unless otherwise stated. The statistical tests used were paired *t* test with Bonferroni corrections in the case of results derived from independent experiments performed in monoplicate (Fig. 1*D*; Fig. 2*A* and *B*, *right panel*; and Fig. 5) and one-way ANOVAs for all other cases.

RESULTS

HDLs protect β-cells against ER stressor–induced apoptosis. To determine whether HDLs antagonize the negative effects of pathophysiological ER stressors or ER stress–inducing drugs on β-cells, we first used thapsigargin (TG) on mouse insulinoma cells, as well as on human and rat islets. TG is a SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor that induces ER calcium depletion and ER stress (35). Apoptosis induced by TG in primary human islet cells was decreased by HDLs (Fig. 1*A*). Similarly, primary rat β-cells were protected by HDLs against TG-induced apoptosis (Fig. 1*B*). In the MIN6 mouse insulinoma cell line, HDLs very efficiently inhibited TG-induced apoptosis as indicated by a reduction in the number of cells with pycnotic nucleus and by a decrease in caspase-3 activation (Fig. 1*C*). HDLs also protected MIN6 cells against death induced by cyclopiazonic acid (CPA), another SERCA inhibitor (Supplementary Fig. 1). Free fatty acids contribute to the development of type 2 diabetes by inducing ER stress, β-cell dysfunction, and apoptosis (36). Palmitate-induced apoptosis was significantly reduced by HDLs (Fig. 1*D*). Hyperglycemia, observed in the diabetic patient, is another factor suggested to induce ER stress in pancreatic islets (37). One week exposure of rat islets to 30 mmol/L glucose almost quadrupled the rate of apoptosis in insulin-expressing cells (Fig. 1*E* and *F*). This was fully prevented by HDLs (Fig. 1*E* and *F*). Altogether,

***A*. *E* and *F*: Cultured rat islets were incubated for a week in serum-free RPMI 1640 medium containing 5 g/L BSA and 10 or 30 mmol/L glucose (labeled G10 and G30 in the figure) in the presence (HDL) or in the absence (vehicle [VEH]) of 1 mmol/L HDLs. Apoptosis was then assessed as in *B*. *E*: representative examples of TUNEL staining (green staining) in insulin-positive cells (red staining; nuclei are stained in blue with DAPI). The corresponding quantitation is shown in *F*. *Significant differences. (A high-quality digital representation of this figure is available in the online issue.)**

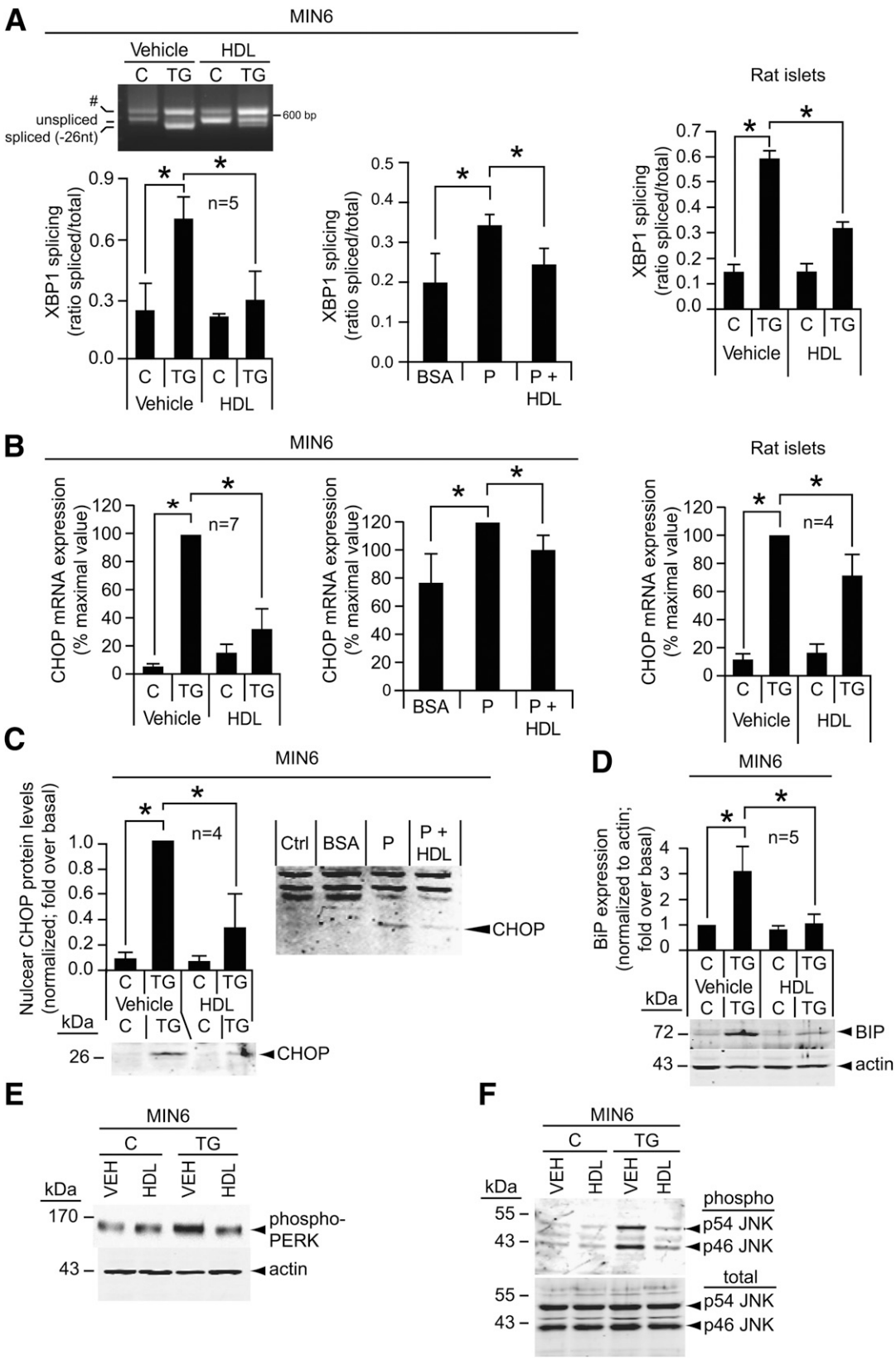

FIG. 2. HDLs inhibit the induction of stress markers by TG and palmitate. MIN6 cells and rat islets were left untreated (control [C]) or treated with 0.5 μmol/L TG during 6 h (*A–C* and *E*) or 24 h (*D* and *F*) in the presence or in the absence of 1 mmol/L HDLs. Alternatively, the cells were treated with 0.3% BSA (BSA) or with 0.3% BSA/0.4 mmol/L palmitate (P) in the presence or in the absence of 1 mmol/L HDLs for 24 h (*A* and *B*) or 48 h (*C*). The cells were then lysed, and RNA and proteins were isolated. The extent of XBP1 mRNA splicing was then determined (*A*). The pound sign (#) in *A* indicates an unspecific band (see RESEARCH DESIGN AND METHODS). CHOP mRNA expression was determined by quantitative PCR (*B*). Western blot analysis were performed to assess protein expression of CHOP (*C*), BiP (*D*), phospho-PERK (*E*), and phospho- and total JNK (*F*). The experiments presented in *E* and *F* were repeated once and twice, respectively, and yielded similar results. *Significant differences. VEH, vehicle.

these results indicate that HDLs protect β-cells against a broad range of pathophysiological ER stressors.

In endothelial cells, HDLs activate the antiapoptotic Akt kinase (38) and this presumably requires binding of HDLs to the scavenger receptor class B, type I (SR-BI) (39,40). In β-cells, SR-BI does not appear to be involved in HDL-induced suppression of IL-1β–mediated apoptosis (24). Whether SR-BI is involved in β-cell protection may depend on the proapoptotic stimulus however. We therefore tested whether this receptor could mediate the protective effect of HDLs against ER stress. MIN6 cells were transfected with siRNA duplexes directed at the SR-BI mRNA. This led to a ~80% reduction in SR-BI protein expression levels (Supplementary Fig. 2*C*). In these conditions, HDLs were still able to protect β-cells against both TG and palmitate-induced apoptosis (Supplementary Fig. 2*A* and *B*). This suggests that SR-BI is dispensable for HDL-mediated β-cell protection.

Specificity of the HDL-induced protection against TG-induced β-cell apoptosis. TG is a lipophilic compound that could potentially bind to HDLs, preventing it from

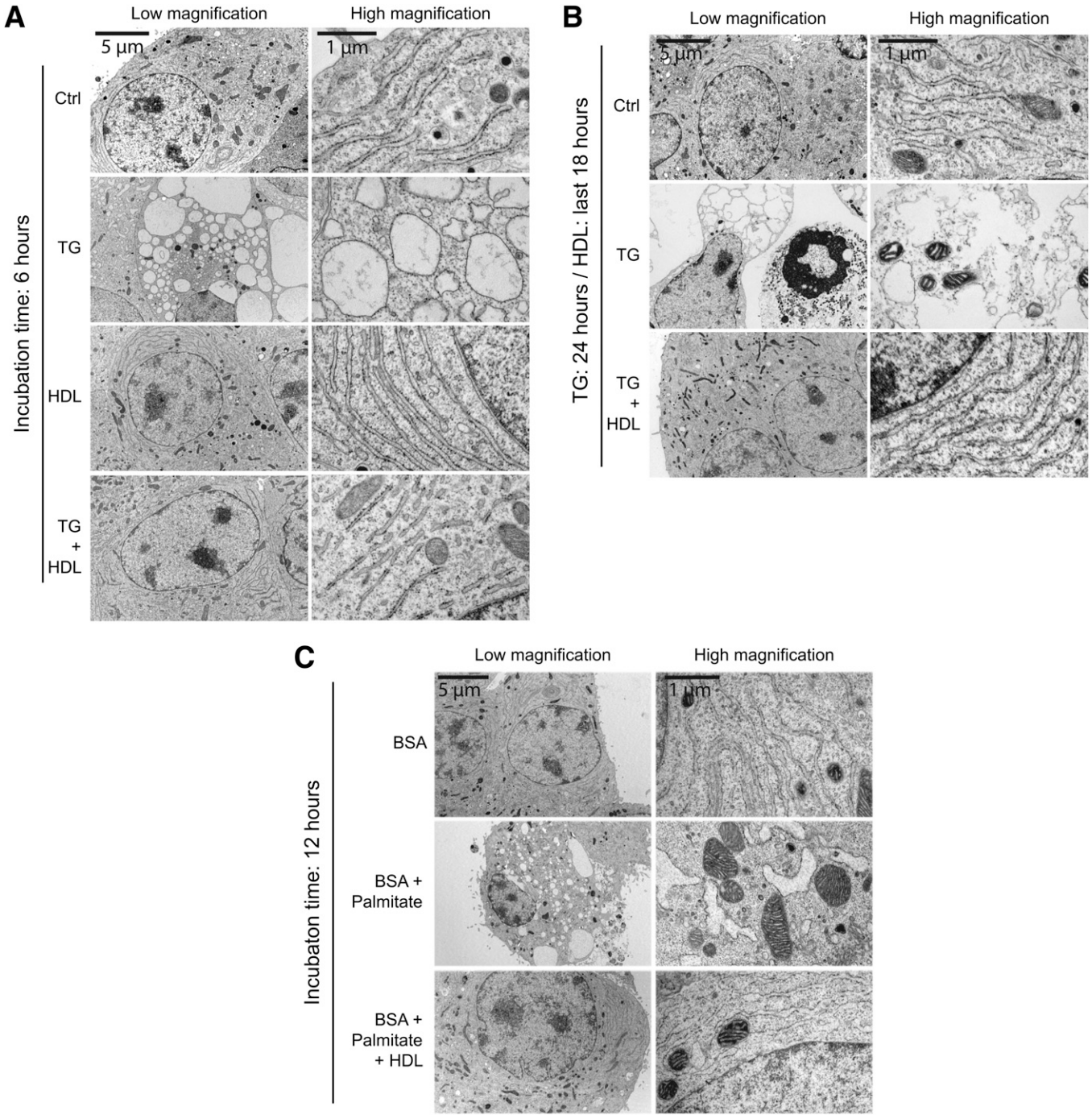

FIG. 3. HDLs prevent TG- and palmitate-induced ER morphology alterations. MIN6 cells were plated on glass slides previously coated with poly-L-lysine and incubated for 6 h with the indicated treatments (*A*). Alternatively, the cells were treated for 6 h with TG and then incubated or not with HDLs for an additional 18 h-period (*B*). *C*: cells were incubated for 12 h with 0.3% BSA, 0.4 mmol/L palmitate, and 1 mmol/L HDLs in the indicated combinations. The cells were then processed for electron microscopy as described in RESEARCH DESIGN AND METHODS. Ctrl, control.

inhibiting SERCA. To determine whether HDLs could affect the SERCA inhibitory activity of TG, cytosolic calcium concentration was monitored by Fura-2 calcium imaging. Supplementary Fig. 3 shows that TG, as expected from its ability to induce ER calcium depletion, stimulated an increase in cytoplasmic calcium. HDLs did not affect this response, indicating that they do not prevent the inhibitory activity of TG on SERCA. We next assessed the specificity of the protective response induced by HDLs in β-cells by testing the capacity of another lipoprotein, the LDL, to prevent TG-induced MIN6 cell apoptosis. LDLs are not toxic for β-cells unless oxidized (22) (compare also the first and third bar in Supplementary Fig. 4). LDLs were unable to protect MIN6 cells from death induced by TG (Supplementary Fig. 4). This demonstrates that the ability of HDLs to protect β-cells from ER stress is specific for this lipoprotein particle and that it is not a general property of lipoproteins.

To further characterize the protective effects of HDLs, MIN6 cells were subjected to different TG incubation protocols before HDL addition. Supplementary Fig. 5*A* (gray bars) shows that HDLs added 10 min after TG still prevented apoptosis of MIN6 cells, indicating that HDLs protect β-cells when TG had already started the depletion in ER calcium stores (that occurs within less than a minute; see Supplementary Fig. 3). However, continuous TG exposure is required to induce efficient apoptosis of MIN6 cells since washing TG after a 10-min incubation period greatly reduced the extent of cell death (Supplementary Fig. 5*A*, black bars). Supplementary Fig. 5*B* shows indeed that TG has to be present for several hours to induce a clear increase in MIN6 cell death. The same experiment as described in Supplementary Fig. 5*A* was then performed but with longer incubation times with TG (Supplementary Fig. 5*C*). In each case, HDLs significantly reduced the extent of apoptosis after TG removal. Finally, in conditions where TG was added at the beginning of the experiment and was not washed away, addition of HDLs at later times still protected β-cells (Supplementary Fig. 5*D*). The degree of protection was still about 70% when HDLs were added 12 h after the addition of TG. Altogether these experiments indicate that HDLs can reverse the proapoptotic effects of TG, which is consistent with their ability to reverse the ER morphological alterations induced by TG (see Fig. 3 below).

HDLs prevent ER stress signaling. TG- and palmitate-induced ER stress in MIN6 cells and primary rat islet cells as assessed by their ability to increase XBP1 mRNA splicing (Fig. 2*A*), CHOP mRNA, and nuclear protein levels (Fig. 2*B* and *C*, respectively), BiP protein levels (Fig. 2*D*), and phosphorylation of PERK (Fig. 2*E*) and JNK (Fig. 2*F*). Induction of all these ER stress markers was significantly inhibited by HDLs. Therefore, the ability of HDLs to prevent β-cell death induced by TG and palmitate correlates with their capacity to dampen ER stress signaling.

HDLs reverse TG-induced ER morphology disruption. As reported previously (41), TG induced extensive dilation of the ER after a 6-h incubation period (compare the first and second rows in Fig. 3*A*). This was prevented by HDLs (fourth row in Fig. 3*A*). A 24-h treatment with TG, in addition to inducing ER swelling, led to the appearance of apoptotic features such as chromatin condensation and pycnosis (Fig. 3*B*). When HDLs were added 6 h after TG, i.e., at a time when ER was extensively dilated (see Fig. 3*A*), and incubated for an additional 18-h period in the presence of TG, ER morphology greatly recovered and the signs of apoptosis disappeared. Palmitate also induced ER dilation, although to a lesser extent than TG; but it is noteworthy that this was efficiently prevented by HDLs (Fig. 3*C*). These results indicate that HDLs not only prevent ER morphology disruption brought about by ER stress but also allow cells with disrupted ER to recover a normal morphology even in the continuous presence of an ER stressor.

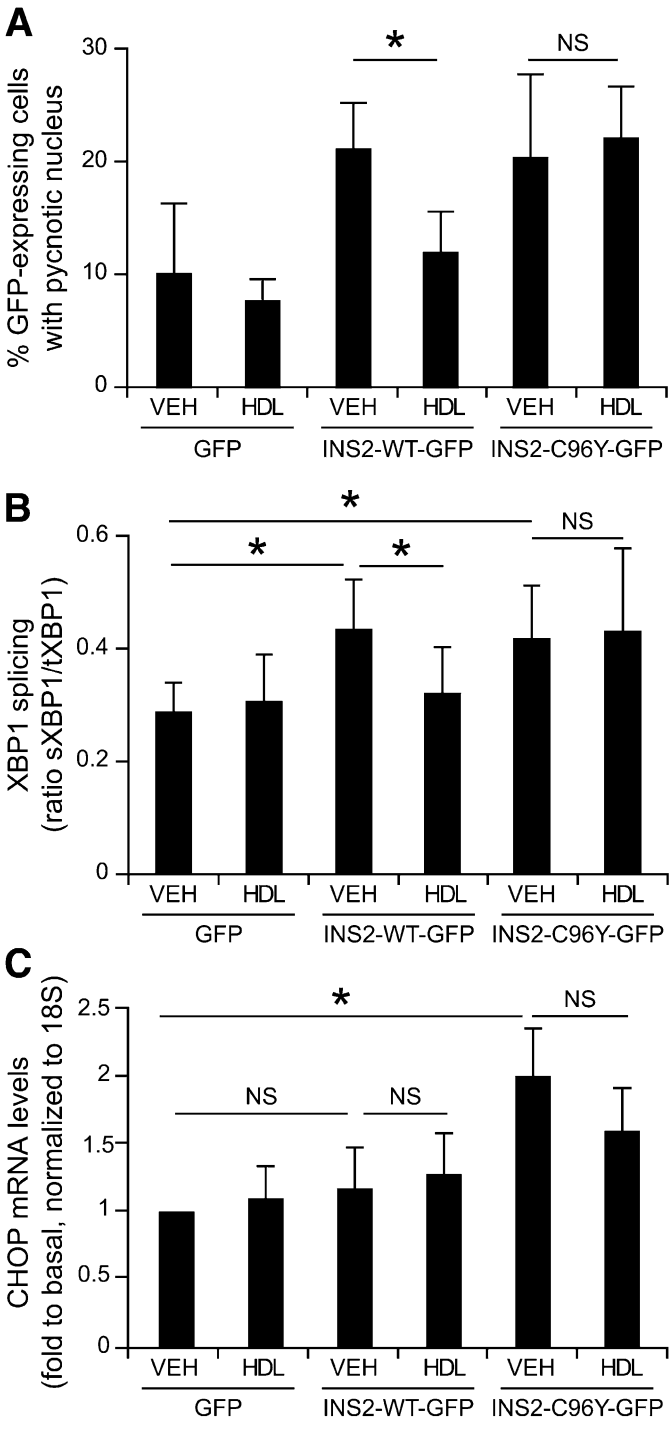

FIG. 4. Insulin overexpression-induced β-cell apoptosis is inhibited by HDLs. MIN6 cells were infected with lentiviruses encoding the indicated constructs. Three days later, cells were trypsinized and plated in new culture dishes for 4 days, the last two days in the presence or in the absence of 1 mmol/L HDLs. Apoptosis was then determined by scoring pycnotic and fragmented nuclei (*A*). Alternatively, 24 h after the infection, the extent of XBP1 mRNA splicing (*B*) and CHOP mRNA expression (*C*) were determined. *indicates significant differences. VEH, vehicle; NS, no significant differences.

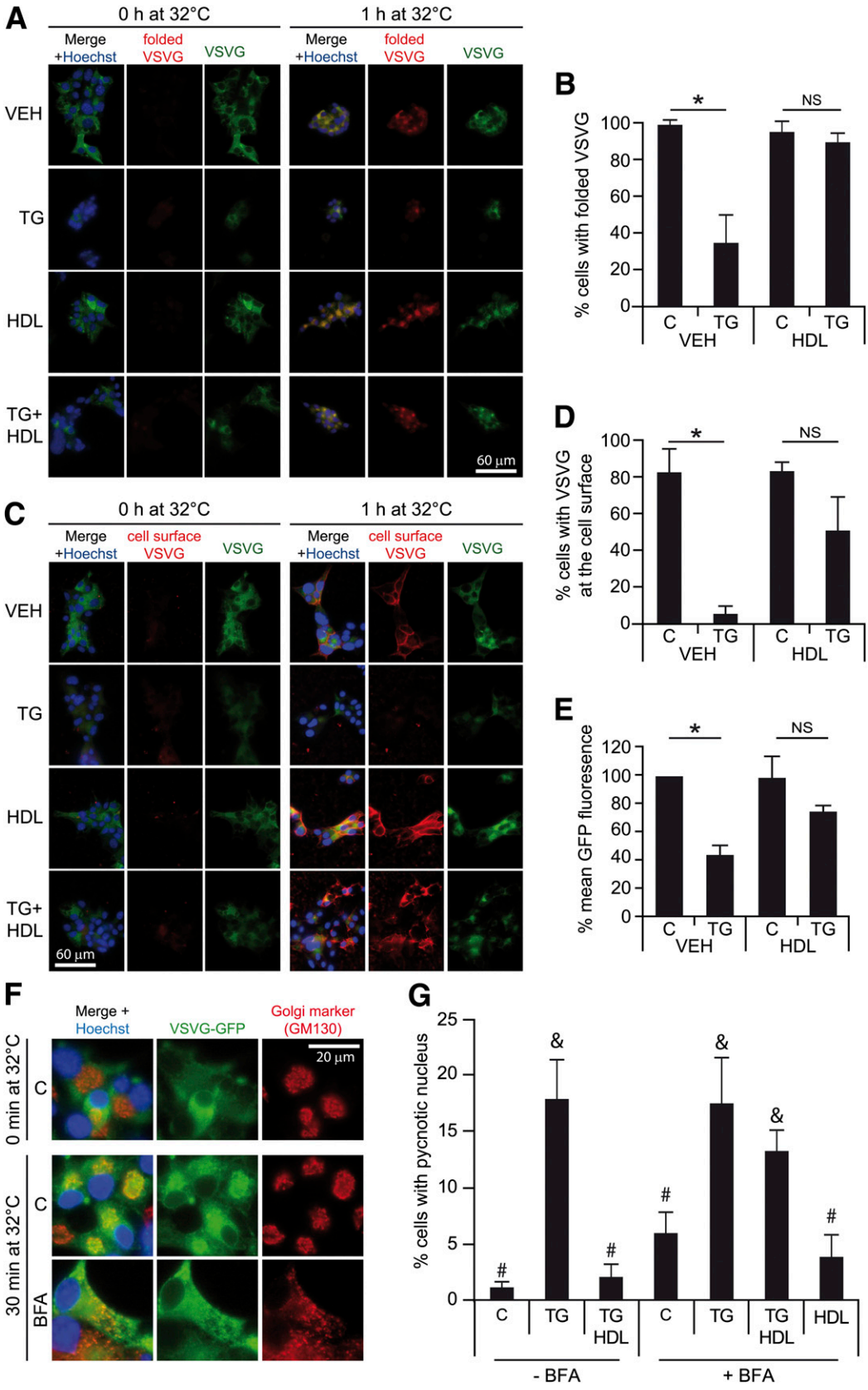

FIG. 5. HDL-mediated β-cell protection against TG-induced apoptosis is inhibited by BFA. *A* and *B*: MIN6 cells were infected with VSVG-GFP-encoding lentiviruses and treated 2 days later with or without 0.5 µmol/L TG in the presence or in the absence of 1 mmol/L HDLs for 5 h at 40°C. The cells were then incubated or not for an additional 1-h time period at 32°C. The presence of folded VSVG was assessed by immunocytochemistry on permeabilized cells using an antibody specifically recognizing the correctly folded form of the protein. The percentage of cells expressing folded VSVG was quantitated and shown in *B*. *C–E*: Cells were treated as in *A* except that nonpermeabilized cells were labeled with an antibody directed against the ectopic part of VSVG. The percentage of cells expressing VSVG at the cell surface was quantitated and shown in *D*. Quantification of the

HDLs improve protein folding and export capacity of the ER. The ability of HDLs to inhibit apoptosis induced by ER stressors could result from an augmented capacity of the ER to fold proteins and favor their export or from an increased capacity to tolerate a given ER stress. As a first approach to distinguish between these two possibilities, we overexpressed GFP fusion proteins with wild-type insulin-2 and a mutant form of insulin-2, found in the Akita mouse, that cannot fold properly because of a cysteine to tyrosine substitution at position 96. This mutation prevents the formation of a disulfide bridge required for insulin maturation (42). Thus the (re)folding mechanisms of the cell cannot correct this altered conformation, leading to sustain ER stress and apoptosis (7), unless downstream proapoptotic effectors, such as CHOP, are inactivated (7). Expression of the insulin-GFP fusion protein in MIN6 cells after lentiviral infection led to a ~60% increase in total insulin content (i.e., endogenous insulin + insulin-GFP; Supplementary Table 2), and this induced a mild but significant apoptotic response (Fig. 4*A*). Expression of the insulin-GFP constructs by transfection similarly induced apoptosis (Supplementary Fig. 6). In each case, the apoptotic response mediated by insulin overexpression was blocked by HDLs. In contrast, apoptosis induced by expression of the insulin C96Y-GFP fusion protein in MIN6 cells was not antagonized by HDLs (Fig. 4*A* and Supplementary Fig. 6). Paralleling these responses, XBP1 mRNA splicing was augmented by both wild-type and mutant insulin, but HDLs only inhibited the splicing of XBP1 mRNA induced by wild-type insulin (Fig. 4*B*). This indicates that overexpression of the C96Y insulin mutant exerts a stress on the ER that may be more severe (or of a different nature) than the one induced by wild-type insulin overexpression. The C96Y insulin mutant appears indeed to induce a stronger burden on the ER than wild-type insulin because it significantly increased CHOP expression, whereas wild-type insulin did not (Fig. 4*C*). The increased CHOP expression after overexpression of the insulin C96Y mutant was not antagonized by HDLs (Fig. 4*C*). One interpretation of the above results is that HDLs do not protect β-cells experiencing an ER stress response that cannot be alleviated such as when cells express an insulin mutant that cannot fold properly.

HDLs may protect β-cells against ER stress by increasing the functionality of the ER eventually dampening the initial ER stress. This is consistent with the ability of HDLs to lower the expression of ER stress markers induced by TG and palmitate (see Fig. 2). To directly assess the capacity of HDLs to favor protein folding and trafficking, we took advantage of a ts mutant of VSVG fused to GFP (32). This mutant cannot fold correctly at 40°C and is therefore retained in the ER. However, at the permissive temperature (32°C), it adopts a correctly folded conformation and can then traffic from the ER to the Golgi and finally to the cell surface. As expected, cells cultured at 32°C expressed the correctly folded VSVG-GFP fusion protein as determined by staining with a conformation-specific anti-VSVG antibody (Cy3-positive cells in Supplementary Fig. 7). At 40°C, no cells expressed the correctly folded VSVG protein whether HDLs were present or not (Supplementary Fig. 7). HDLs were not able to increase the VSVG folding capacity of the cells at 39°C, a temperature that allowed partial folding of VSVG (Supplementary Fig. 7). Possibly, VSVG misfolding induced by high temperatures is intrinsically irreversible, akin to the misfolding of the C96Y insulin mutant.

To determine whether HDLs can favor protein folding and trafficking in the presence of an ER stressor, we evaluated the influence of HDLs on VSVG folding and trafficking in conditions where HDLs can inhibit apoptosis. MIN6 cells were incubated or not with TG in the presence or absence of HDLs (Fig. 5). A 5-h incubation at 40°C led to accumulation of misfolded VSVG in the ER (Fig. 5*A*, *left panel*). Releasing this block by placing the cells at 32°C allowed VSVG to be correctly folded (Fig. 5*A*, *right panel*). This was prevented by TG indicating that this SERCA inhibitor impairs protein folding. HDLs, however, restored the capacity of the cells to correctly fold the VSVG protein in the presence of TG (Fig. 5*A* and *B*). Protein trafficking was assessed by monitoring the appearance of VSVG at the cell surface after the release of the cells from the 40°C block. TG did not allow VSVG cell surface appearance (Fig. 5*C*, *right panel*). This was reversed by HDLs (Fig. 5*C* and *D*). HDLs can therefore inhibit the capacity of TG to hamper the correct folding of VSVG and its trafficking from the ER to the surface of the cells. We also noted that TG decreased the expression of the VSVG-GFP fusion protein (Fig. 5*C–E*) indicating that this ER stressor also perturbs protein synthesis possibly as a result of ER homeostasis alteration. This again was not occurring in the presence of HDLs (Fig. 5*C–E*).

The causality between the ability of HDLs to efficiently protect β-cells against ER stress and their capacity to improve the trafficking of proteins was investigated using brefeldin A (BFA). This compound is able to inhibit the trafficking of protein from the ER to the Golgi (43). Hence as expected, BFA blocked the translocation of VSVG from the ER to the Golgi (Fig. 5*F*). Figure 5*G* shows that BFA prevented HDLs from inhibiting TG-induced β-cell apoptosis. This indicates that the capacity of HDLs to promote protein trafficking in the presence of an ER stress is required for their antiapoptotic ability.

We next analyzed the ability of HDLs to prevent ER protein export perturbation induced by palmitate. Palmitate was shown earlier to impair ER to Golgi trafficking (44). However, palmitate does not reduce VSVG folding (44). Thirty minutes after the release from the 40°C block, about 50% of the cells had the majority of their VSVG proteins move from the ER to the Golgi (Fig. 6). ER export of VSVG was reduced by palmitate but this was antagonized by HDLs (Fig. 6*C*). As reported previously, palmitate did not affect VSVG folding (Fig. 6*D*). It is noteworthy that the presence of HDLs alone was able to slightly, but significantly, increase the number of correctly folded VSVG. This suggests that HDLs contribute to improve β-cell ER homeostasis by favoring both protein folding and trafficking.

HDL oxidation is one alteration that is thought to occur in diabetes or in the metabolic syndrome (45). Therefore, it was of interest to assess the capacity of oxidized HDLs

GFP fluorescence intensity in VSVG-GFP expressing cells is presented in *E*. *F* and *G*: MIN6 cells infected with VSVG-GFP-encoding lentiviruses were preincubated or not with 250 ng/mL BFA for 2 h before being subjected to the indicated combinations of 0.5 μmol/L TG and 1 mmol/L HDLs for an additional 22-h period. Permeabilized cells were then stained with an antibody recognizing GM130, a specific Golgi marker (*F*). Alternatively, apoptosis was assessed by scoring cells with pycnotic and/or fragmented nucleus (*G*). Means with different symbol (# or &) are significantly different. Nuclei were stained in blue with the Hoechst 33342 dye. *Significant differences. NS, no significant differences. VEH, vehicle; C, control. (A high-quality digital representation of this figure is available in the online issue.)

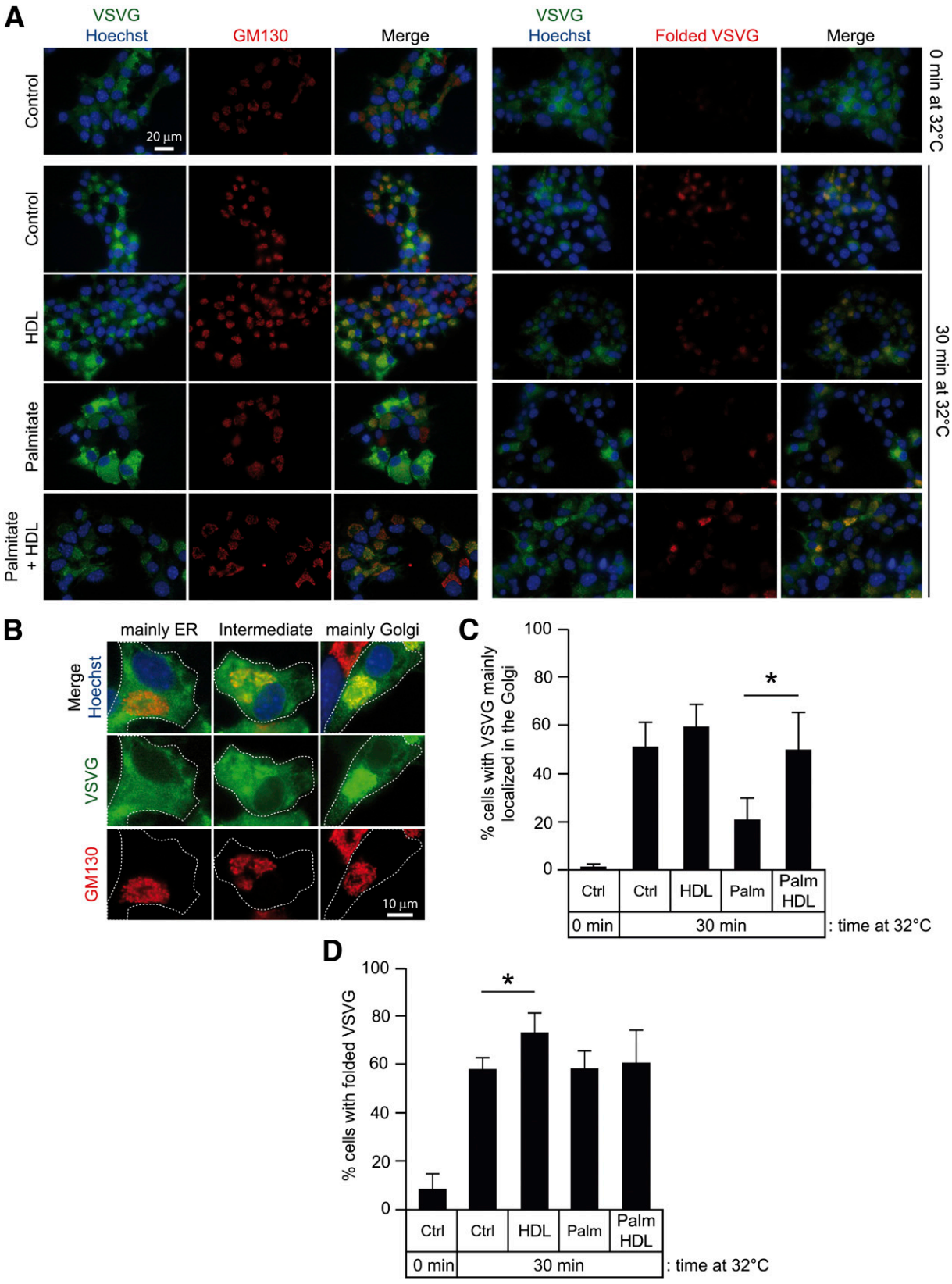

FIG. 6. HDLs restore ER to Golgi trafficking in palmitate (Palm)-treated cells. MIN6 cells were infected with VSVG-GFP-encoding lentiviruses and treated or not with 0.4 mmol/L palmitate in the presence or in the absence of 1 mmol/L HDLs for 48 h (in each case, BSA was present at a 0.3% concentration). The cells were then incubated 5 h at 40°C. They were then treated for 15 min with 5 μmol/L cycloheximide before switching the temperature to 32°C for 0 or 30 min (note that the temperature shifts and/or cycloheximide did not induce apoptosis; see Supplementary Fig. 8). The cells were stained with an antibody recognizing the Golgi marker GM130 (red staining; left part of *A*) or processed as described in Fig. 5*A* and *B* (right part of *A*). Nuclei were stained in blue with the Hoechst 33342 dye. Representative examples of the different locations of VSVG in cells are shown in *B* (VSVG mainly in the ER when the GFP signal does not colocalize with GM130 staining; VSVG mainly in the Golgi when these two signals extensively colocalize; and intermediate situation when the GFP signal only partially colocalizes with the Golgi marker). *C*: the quantitation of the

to protect β-cells against ER stress–induced apoptosis. Figure 7 shows that, in contrast with native HDLs, oxidized HDLs were not able to antagonize TG-induced apoptosis of MIN6 cells.

DISCUSSION

In the current study, HDLs were found to efficiently inhibit apoptosis induced by several ER stressors (Fig. 1). This was observed both in insulinoma cells and in primary human and rat islet β-cells. This protection was associated with a diminished activation of ER stress markers including XBP1 mRNA splicing, CHOP induction (both at the RNA and protein level), BiP induction, PERK activation, and JNK MAPK stimulation (Fig. 2). A series of control experiments was performed to assess the possibility that HDLs could exert their protective action against ER stressors (TG in particular) by merely sequestering them away from cells. This possibility could be excluded because of the following observations. HDLs did not prevent TG from inducing an increase in cytoplasmic calcium concentration after its inhibitory action on SERCA (Supplementary Fig. 3). LDL, a similar amphipathic lipoprotein, was unable to mimic the antiapoptotic properties of HDLs (Supplementary Fig. 4). Addition of HDLs several hours after TG still inhibited β-cell apoptosis (Supplementary Fig. 4) and was able to restore a normal ER morphology (Fig. 3). HDLs prevented β-cell apoptosis and ER stress marker induction in response to chemically unrelated substances and treatments (palmitate, CPA, TG, insulin overexpression) (Figs. 1, 2, and 4 and Supplementary Fig. 1). Finally, if HDLs sequestered TG away from cells, they would be expected to always protect cells from TG-induced apoptosis. However, this is not the case since HDLs do in fact lose their capacity to inhibit TG-induced β-cell apoptosis in certain experimental conditions (e.g., when cells are treated with BFA; see Fig. 5*F* and *G*).

HDLs are known to induce antiapoptotic pathways in various cell types. This has been well studied in endothelial cells where HDLs stimulate the antiapoptotic Akt kinase (38). EDG receptors and the scavenger receptor SR-BI are likely to mediate the HDL effects in these cells (39,40,46). In contrast, the signaling pathways mediating HDL-induced antiapoptotic response in β-cells are not known. Furthermore, SR-BI does not seem to be involved in HDL-mediated protection of IL-1β–treated β-cells (24,47) or against TG- and palmitate-induced death (Supplementary Fig. 2). The paucity of information on the signaling pathways that are involved in the protective function induced by HDLs in β-cells contrasts with the numerous data showing beneficial effects of HDLs against diabetes in rodents and humans.

Our results show that HDL-mediated β-cell protection correlates with ER morphology preservation, as well as maintenance of a functional protein folding in the ER and protein export from the ER. However, when protein transport from the ER to the Golgi was inhibited with BFA, HDLs lost their protective effect (Fig. 5*F* and *G*), indicating that the maintenance of a proper ER secretory capacity is required for HDL-induced inhibition of ER stressor–induced β-cell apoptosis. HDL-mediated protection of β-cells via improved protein folding and ER export is the first cellular mechanism to be identified explaining how HDLs protect β-cells against prodiabetogenic factors such as FFAs, high glucose concentrations, and ER overload. Improved functionality of the ER could also participate in the known ability of HDLs to ameliorate the insulin secretory capacity of the β-cell (19,25). Our data suggest that improved ER functionality is an underlying mechanism counteracting the development of diabetes during interventions to increase HDL levels in humans exposed to prodiabetogenic factors. Our findings are in line with the notion that alleviating ER stress is a new approach to treat diabetes (48). We have observed that oxidation of HDLs abrogates their ability to protect β-cells against ER stress–induced apoptosis (Fig. 7). Therefore, one of the contributions to accelerated β-cell mass decrease in diabetic patients, who have increased levels of oxidized HDLs (45), may be an inefficient HDL-mediated protection of β-cells against ER stress. Hence, means of preserving HDLs integrity and functionality represent potential avenues of treatments against diabetes.

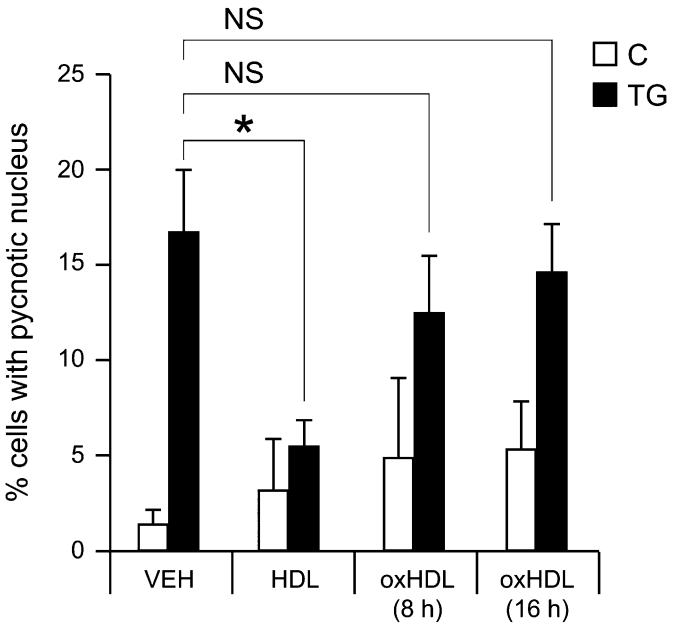

FIG. 7. Oxidized HDLs (oxHDL) do not protect β-cells against TG-induced apoptosis. MIN6 cells were treated with 0.5 μmol/L TG in the presence or in the absence of 1 mmol/L nonoxidized or oxidized HDLs (oxidation performed during 8 or 16 h) for 24 h. Cells were then fixed, and apoptosis was assessed by scoring pycnotic nuclei. *Significant differences. VEH, vehicle; NS, no significant differences; C, control.

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J.Pé., G.W., and C.W. designed the experiments. J.Pé. and C.W. cowrote the manuscript. J.D. and J.-C.J. performed

percentage of cells with VSVG mainly localized in the Golgi, and *D* depicts the quantitation of the percentage of cells with correctly folded VSVG (results derived from 5 independent experiments each). *Significant differences. Ctrl, control. (A high-quality digital representation of this figure is available in the online issue.)

experiments on rat islets J.Pu. performed electron microscopy. J.-Y.C. performed fluorescence calcium imaging. M.F. and R.W.J. performed the oxidation of HDLs. F.A. quantitated insulin. J.Pé. performed all other experiments. All authors discussed the results and edited the manuscript. J.Pé. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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