

# MafA Expression and Insulin Promoter Activity Are Induced by Nicotinamide and Related Compounds in INS-1 Pancreatic $\beta$ -Cells

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Nicotinamide has been reported to induce differentiation of precursor/stem cells toward a  $\beta$ -cell phenotype, increase islet regeneration, and enhance insulin biosynthesis. Exposure of INS-1  $\beta$ -cells to elevated glucose leads to reduced insulin gene transcription, and this is associated with diminished binding of pancreatic duodenal homeobox factor 1 (PDX-1) and mammalian homologue of avian MafA/L-Maf (MafA). Nicotinamide and other low-potency poly(ADP-ribose) polymerase (PARP) inhibitors were thus tested for their ability to restore insulin promoter activity. The low-potency PARP inhibitors nicotinamide, 3-aminobenzamide, or PD128763 increased expression of a human insulin reporter gene suppressed by elevated glucose. In contrast, the potent PARP-1 inhibitors PJ34 or INO-1001 had no effect on promoter activity. Antioxidants, including N-acetylcysteine, lipoic acid, or quercetin, only minimally induced the insulin promoter. Site-directed mutations of the human insulin promoter mapped the low-potency PARP inhibitor response to the C1 element, which serves as a MafA binding site. INS-1 cells exposed to elevated glucose had markedly reduced MafA protein and mRNA levels. Low-potency PARP inhibitors restored MafA mRNA and protein levels, but they had no effect on PDX-1 protein levels or binding activity. Increased MafA expression by low-potency PARP inhibitors was independent of increased MafA protein or mRNA stability. These data suggest that low-potency PARP inhibitors increase insulin biosynthesis, in part, through a mechanism involving increased MafA gene transcription. *Diabetes* 55:742–750, 2006

**A**cute elevations in glucose concentration regulate pancreatic  $\beta$ -cell function, including stimulation of insulin secretion and biosynthesis. In contrast, chronic exposure of  $\beta$ -cells to elevated glucose diminishes cell function by altering  $\beta$ -cell

gene expression (1) through a poorly understood process termed glucose toxicity. One of the associated outcomes of glucose toxicity on  $\beta$ -cells is a reduction in insulin gene expression (1–6) that results from decreased insulin promoter activity (2–4,7–9). The decline in insulin promoter activity correlates to a reduction in expression and/or binding of  $\beta$ -cell-enriched transcription factors, including pancreatic duodenal homeobox factor 1 (PDX-1) (1,4–6,8,10) and mammalian homologue of avian MafA/L-Maf (MafA; also known as RIPE3b1 or C1 activator) (3,4,7,9,11). The biochemical mechanisms whereby elevated glucose reduced PDX-1 and MafA binding activity are unknown; however, a number of studies support the possible involvement of oxidative stress (6,11–14).

Nicotinamide has historically been used to augment pancreatic  $\beta$ -cell differentiation and protect islet cells from toxic insults. Treatment of cultured fetal human or porcine islet-like cell clusters, pancreatic precursor/stem cells, or embryonic stem cells with nicotinamide enhances the differentiation of  $\beta$ -cells, and this is characterized by increased insulin mRNA levels, biosynthesis, and intracellular content (15–18). In vivo, nicotinamide has been shown to increase  $\beta$ -cell replication in transplanted islets (19), stimulate  $\beta$ -cell regeneration in partially pancreatectomized rats (20), lengthen the “honeymoon” period in type 1 diabetic patients (21), and improve insulin secretion from patients at high risk of developing type 1 diabetes (22). Nicotinamide also protects  $\beta$ -cells from streptozotocin-induced damage by competitive inhibition of poly(ADP-ribose) polymerase (PARP) (23), suggesting that nicotinamide may influence  $\beta$ -cell differentiation and regeneration through a mechanism involving inhibition of PARP. Consistent with this possibility, the PARP inhibitor 3-aminobenzamide increases  $\beta$ -cell regeneration and prevents diabetes in partially pancreatectomized rats (20).

Prolonged exposure of INS-1  $\beta$ -cells to elevated glucose leads to reduced insulin gene expression and promoter activity, and this is associated with decreased binding activity of PDX-1 and MafA (4). Overexpression of MafA, but not PDX-1, strongly induced insulin promoter activity in INS-1 cells cultured in elevated glucose (9). These data suggested that loss of MafA binding activity is a primary cause for glucose-mediated suppression of insulin promoter activity. Studies described herein show that low-potency PARP inhibitors, including nicotinamide, 3-aminobenzamide, and PD128763, but not potent PARP-1 inhibitors or antioxidants, induce insulin gene expression and promoter activity in INS-1  $\beta$ -cells. The ability of low-potency PARP inhibitors to

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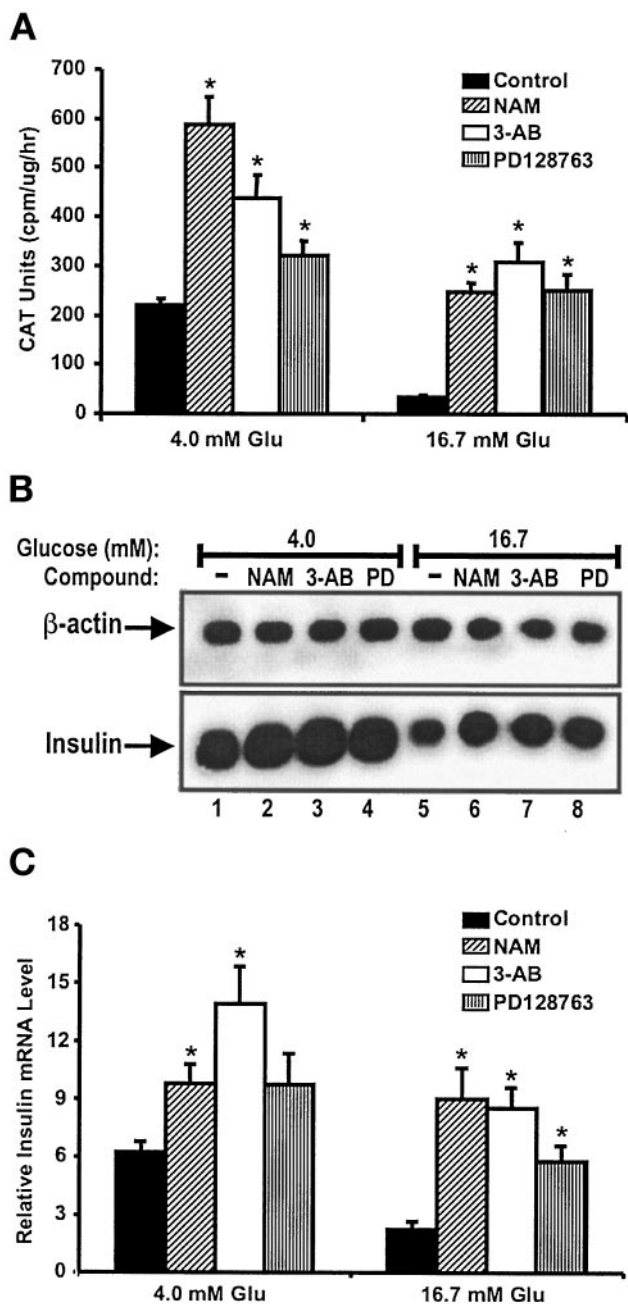
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BETA2,  $\beta$ -cell E-box trans-activator 2; CAT, chloramphenicol acetyltransferase; DCF, dichlorofluorescein; H<sub>2</sub>DCF, dihydrodichlorofluorescein; MafA, mammalian homologue of avian MafA/L-Maf; NAC, N-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PDX-1, pancreatic duodenal homeobox factor 1; ROS, reactive oxygen species.

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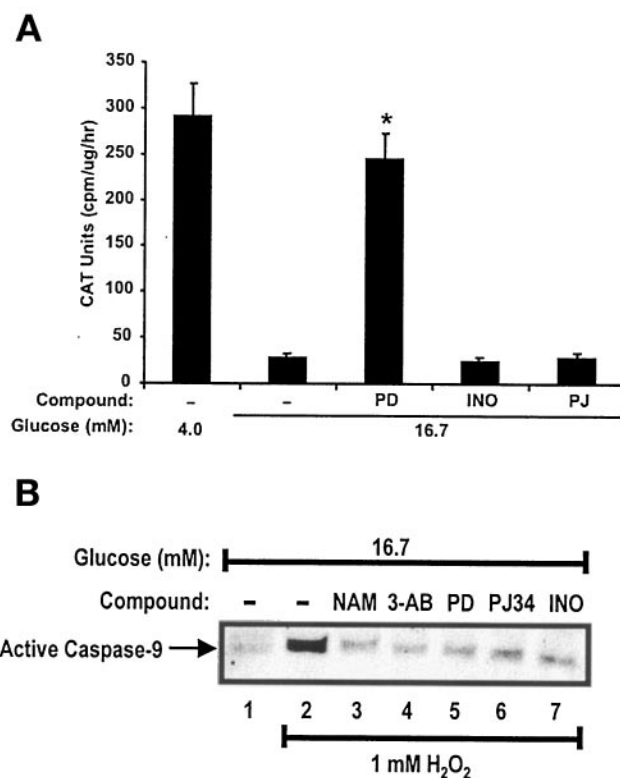
**FIG. 1.** Low-potency PARP inhibitors attenuated glucose suppression of insulin mRNA levels and promoter activity. **A:** INS(-327)CAT expression in INS-1 cells cultured for 48 h in 4 or 16.7 mmol/l glucose (Glu) and vehicle, nicotinamide (NAM; 10 mmol/l), 3-aminobenzamide (3-AB; 10 mmol/l), or PD128763 (PD; 500  $\mu$ mol/l) ( $n = 6$ ). **B:** Insulin and  $\beta$ -actin mRNA levels in INS-1 cells cultured as described above. **C:** Insulin mRNA levels relative to  $\beta$ -actin mRNA levels ( $n = 9$ ). \* $P < 0.05$  for control vs. compounds.

increase insulin gene promoter activity involved a mechanism consistent with increased MafA gene transcription.

#### RESEARCH DESIGN AND METHODS

Anti-PDX-1 antibodies were from Dr. Wright, Vanderbilt University (Nashville, TN). Anti-MafA antibodies (BL1069) were from Bethyl Laboratories (Montgomery, TX). Cleaved caspase-9 antibody was from Cell Signaling Technology (Beverly, MA). PD128763 was from Pfizer (Groton, CT). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz (Santa Cruz, CA).

**Cell culture.** INS-1 cells were maintained in RPMI-1640 media containing 11.1 mmol/l glucose and supplemented with 10% fetal bovine serum, 1 mmol/l



**FIG. 2.** Potent PARP-1 inhibitors are ineffective at restoring insulin promoter activity suppressed by elevated glucose. **A:** INS(-327)CAT expression in INS-1 cells cultured for 48 h in 4 or 16.7 mmol/l glucose and vehicle, nicotinamide (NAM; 10 mmol/l), 3-aminobenzamide (3-AB; 10 mmol/l), or PD128763 (PD; 500  $\mu$ mol/l), INO-1001 (INO; 3  $\mu$ mol/l), or PJ34 (PJ; 5  $\mu$ mol/l) ( $n = 9$ ). \* $P < 0.0005$ , vehicle vs. compounds. **B:** Effect of PARP inhibitors on H<sub>2</sub>O<sub>2</sub>-induced caspase 9 cleavage as determined by Western analysis ( $n = 3$ ). 3-AB, 3-aminobenzamide; NAM, nicotinamide.

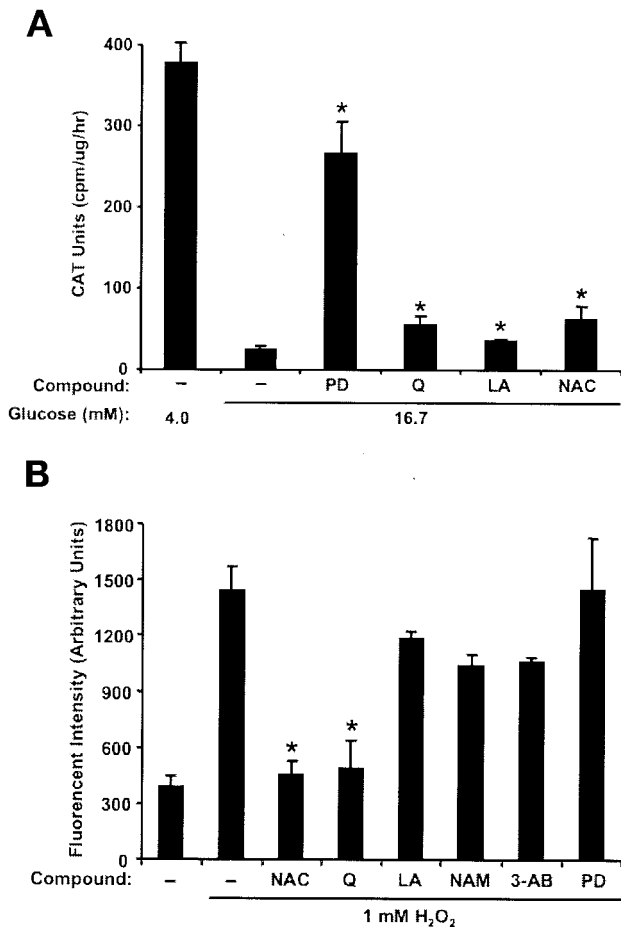
pyruvate, 10 mmol/l HEPES, 50  $\mu$ mol/l 2-mercaptoethanol, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (INS-1 medium).

**Reporter genes studies.** INS(-327)CAT and INS(-230)CAT contain the chloramphenicol acetyltransferase (CAT) gene under transcriptional regulation by the human insulin gene sequences -327/+30 or -230/+30, respectively (9). Site-specific mutations of A1, A3, and C1 elements were generated by PCR amplification using INS(-230)CAT as a template, as previously described (9). Upstream primers used for generation of A1, A3, and C1 mutations were 5'-CCACCCAGGCCCTCCTGGCCAGGCGCA-3', 5'-GC GTCTAGACCCCTGGTTAAGACTCTCCTGACCCGCTGG-3', and CCGGAAAT TGCAGCTGCAGCCCCAGCCATCTG-3', respectively. Mutated bases are bolded and italicized, and underlined sequence indicates restriction enzyme site used for insertion. All plasmid sequences were verified by sequencing on an ABI Prism 3700 DNA analyzer.

INS-1 cells were plated at a density of  $1.5 \times 10^6$  cells per well (diameter 3.5 cm) and subcultured for 2 days in INS-1 medium (11.1 mmol/l glucose). Cells were transfected using a ratio of 1  $\mu$ g plasmid to 2  $\mu$ l lipofectamine and then cultured for 48 h in media containing the indicated glucose concentrations and compounds. CAT assays were performed as previously described (4).

**Northern blot analysis.** Cells were plated at a density of  $5 \times 10^6$  cells per 60-mm dish and subcultured for 2 days in INS-1 medium. Cells were then cultured for various time points in media containing the indicated glucose concentrations and compounds. RNA extraction and Northern blots were performed as previously described (4). Blots were probed with <sup>32</sup>P-labeled cDNAs for human  $\beta$ -actin, MafA, and Syrian hamster preproinsulin (4) and quantified by phosphorimaging.

**Nuclear extracts and electrophoretic mobility shift assays.** Cells were plated at a density of  $10 \times 10^6$  cells per 100-mm dish and subcultured for 2 days in INS-1 medium. Cells were then cultured for various time points in media containing the indicated glucose concentrations and compounds. Nuclear extracts were made as previously described (4,24), except that a phosphatase inhibitor cocktail (10 mmol/l NaF, 1 mmol/l sodium pyrophosphate, 100  $\mu$ mol/l  $\beta$ -glycerol phosphate, and 1 mmol/l sodium orthovanadate) was added to buffers A and B. Double-stranded oligodeoxynucleotide probes to rat insulin 2 gene A2C1 elements (-129 AGCTTGAAACTGCAGCT



**FIG. 3. Antioxidants only minimally restored insulin promoter activity suppressed by elevated glucose. A:** INS(-327)CAT expression in INS-1 cells cultured for 48 h in 4 or 16.7 mmol/l glucose and vehicle, PD128763 (PD; 500  $\mu$ mol/l), quercetin (Q; 10  $\mu$ mol/l),  $\alpha$ -lipoic acid (LA; 1 mmol/l), or NAC (10 mmol/l) ( $n = 4-12$ ). **B:** Effect of compounds on  $H_2O_2$ -induced ROS production ( $n = 4$ ). \* $P < 0.05$ , vehicle plus  $H_2O_2$  vs. compounds plus  $H_2O_2$ . 3-AB, 3-aminobenzamide.

TCAGCCCCTCTGAGCT -96) and to human A3 elements (-230 CCCCTGGT TAAGACTCTAATGACCCGCTGG -201) were labeled with [ $\alpha$ - $^{32}$ P]dCTP. Binding reactions (20-30  $\mu$ g protein per lane) and electrophoresis were performed as described by Shih and Towle (25) and modified by Olson et al. (4). Competition analyses used 100-fold molar excess of unlabeled probe. MafA supershift analyses used 4  $\mu$ g of anti-MafA antibodies, as recommended by the manufacturer.

**Western blot analysis.** For whole-cell lysates, cells were washed in ice-cold PBS, detached by scraping, and pelleted (800g for 3 min at 4°C). Cells were then lysed for 30 min in ice-cold lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 1.5 mmol/l MgCl<sub>2</sub>, 2 mmol/l EGTA, 1% Triton X-100, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 0.5  $\mu$ g/ml leupeptin, and phosphatase inhibitor cocktail), and supernatants were obtained by centrifugation (16,000g for 30 min at 4°C). Nuclear extracts (30 or 60  $\mu$ g) or whole-cell lysates (100  $\mu$ g) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (4). MafA, PDX-1, or cleaved caspase 9 immunoreactivity was detected with specific antibodies and visualized with a SuperSignal West Pico chemiluminescent kit (Pierce).

**Caspase 9 cleavage.** PARP-1 activation has been shown to be a proximal step to caspase activation for  $H_2O_2$ -induced cell death (26). Therefore, the efficacy of PARP inhibitors was determined by examining their ability to attenuate  $H_2O_2$ -induced cleavage of caspase 9. INS-1 cells were plated at a density of  $5 \times 10^6$  cells per 60-mm dish and subcultured for 2 days in INS-1 medium. Cells were pretreated for 1.5 h with media containing 16.7 mmol/l glucose and PARP inhibitors and then incubated for 1 h with 1 mmol/l  $H_2O_2$ . Whole-cell lysates were isolated, and cleavage of caspase 9 was determined by Western blot.

**Dihydrodichlorofluorescein oxidation assay for reactive oxygen species.** Dihydrodichlorofluorescein ( $H_2DCF$ ) oxidation assays modified from the method of Boulares et al. (27) were used to determine the efficacy of PARP

inhibitors to serve as antioxidants. INS-1 cells, cultured to confluency on 96-well dishes, were incubated for 1.5 h with media containing 16.7 mmol/l glucose and indicated compounds. Cells were then incubated for 1 h with 50  $\mu$ mol/l of the diacetate form of  $H_2DCF$  ( $H_2DCFDA$ ; Molecular Probes). Cells were washed and incubated with 1 mmol/l  $H_2O_2$  and indicated compounds. Intracellular reactive oxygen species (ROS) oxidize nonfluorescent  $H_2DCF$  to fluorescent dichlorofluorescein (DCF), which was measured at excitation and emission wavelengths of 485 and 530 nm, respectively.

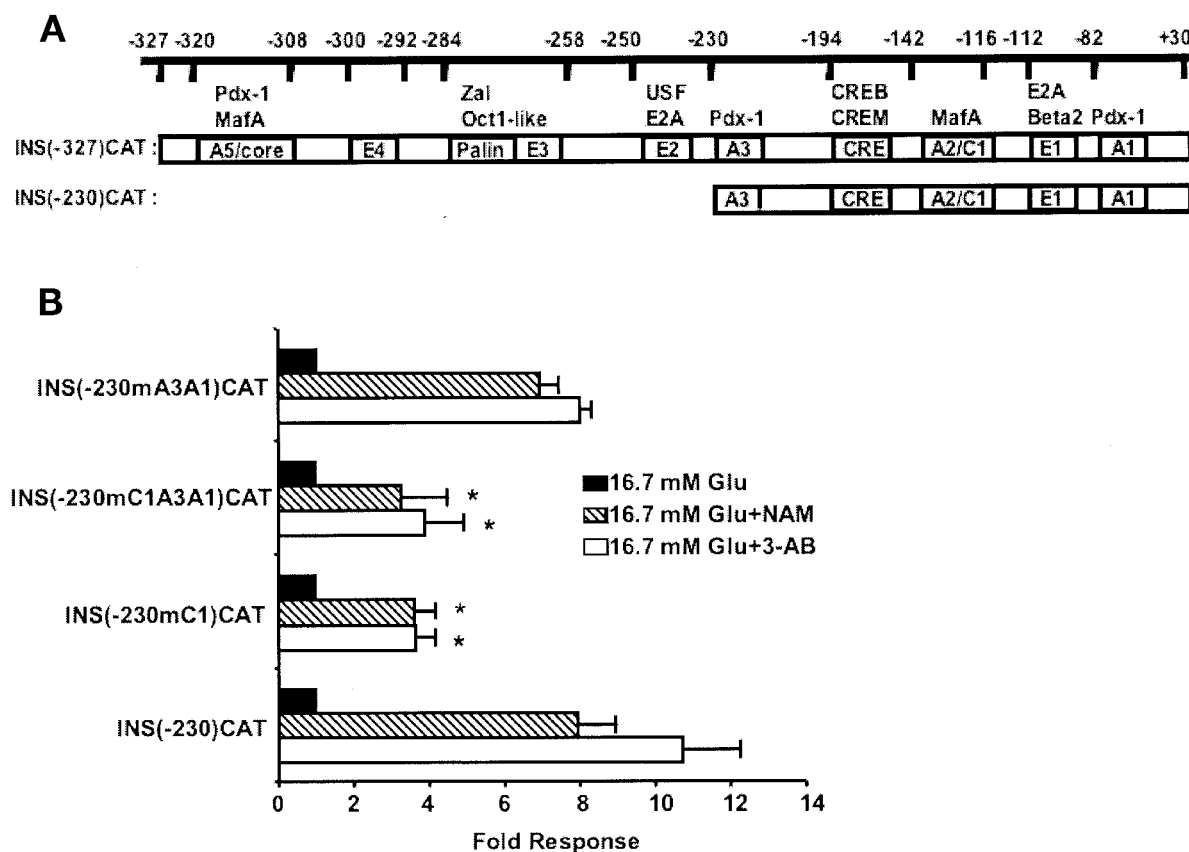
**MafA protein and mRNA stability.** Cells were plated and subcultured as described above for Northern and Western blot analyses. For MafA mRNA stability, cells were then cultured for 24 h in INS-1 medium containing 16.7 mmol/l glucose and 10 mmol/l 3-aminobenzamide, and then they were cultured for the indicated lengths of time in media containing 1  $\mu$ g/ml actinomycin D with or without 10 mmol/l 3-aminobenzamide. Total RNA was isolated and MafA mRNA levels determined by Northern analysis. For MafA protein stability, cells were cultured for 12 h in 4 mmol/l glucose and then for the indicated lengths of time in media containing 40  $\mu$ mol/l cycloheximide with or without 10 mmol/l 3-aminobenzamide. Nuclear extracts were prepared and MafA protein levels assessed by Western analysis.

**Data presentation and statistical analysis.** Data are presented as the means  $\pm$  SE. Comparisons were performed by unpaired Student's *t* test, and  $P < 0.05$  was considered significant.

## RESULTS

**Nicotinamide, 3-aminobenzamide, or PD128763 induce insulin gene promoter activity and insulin mRNA levels.** Low-potency PARP inhibitors, including nicotinamide, 3-aminobenzamide, and PD128763 (28), were tested for the ability to induce insulin gene expression and promoter activity in INS-1 cells. Treatment of INS-1 cells for 48 h with 16.7 mmol/l glucose led to 84% reduction ( $n = 6$ ,  $P < 0.0005$ ) in expression of the human insulin promoter reporter gene INS(-327)CAT (Fig. 1A). Insulin promoter activity reduced by elevated glucose was stimulated seven- to ninefold by treatment of cells with low-potency PARP inhibitors. Low-potency PARP inhibitors also increased insulin promoter activity in cells treated with 4 mmol/l glucose. Low-potency PARP inhibitors had minimal effect on CAT reporter genes regulated by the thymidine kinase gene promoter or the Rous sarcoma virus promoter (data not shown). Treatment of cells with 16.7 mmol/l glucose also suppressed insulin mRNA levels ( $n = 9$ ,  $P < 0.005$ ), and this was partially attenuated by the low-potency PARP inhibitors (Fig. 1B and C). Low-potency PARP inhibitors also increased insulin mRNA levels in cells incubated in 4 mmol/l glucose. Levels of  $\beta$ -actin mRNA were unaffected by treatment of cells with low-potency PARP inhibitors. Because the low-potency PARP inhibitor effect was greatest in INS-1 cells cultured in elevated glucose, subsequent studies with low-potency PARP inhibitors focused on cells cultured in 16.7 mmol/l glucose.

**Potent PARP-1 inhibitors failed to attenuate glucose-mediated suppression of insulin promoter activity.** Two potent PARP-1 inhibitors, PJ34 or INO-1001, were tested for their ability to attenuate glucose suppression of insulin promoter activity. INS-1 cells cultured in 16.7 mmol/l glucose suppressed insulin promoter activity, and this was attenuated by PD128763 (Fig. 2A). In contrast, PJ34 (5  $\mu$ mol/l) or INO-1001 (3  $\mu$ mol/l) had no effect on glucose suppression of insulin promoter activity. In a similar manner, PJ34 had no effect on insulin promoter activity in cells cultured in 4 mmol/l glucose (data not shown). Next, PARP inhibitors were tested for functional activity in INS-1 cells by examining their ability to attenuate  $H_2O_2$ -induced caspase 9 activation/cleavage. PARP-1 activation has been shown to be a proximal step to caspase activation for  $H_2O_2$ -induced cell death (26). For



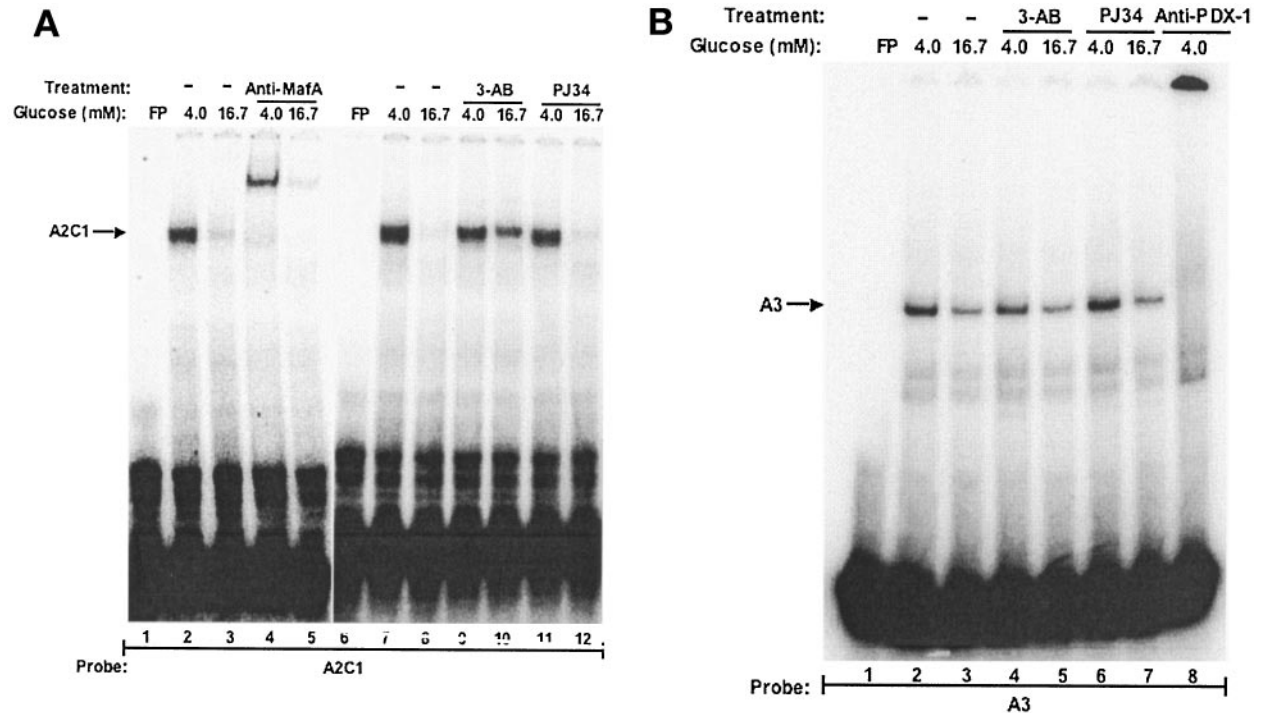
**FIG. 4.** Activation of insulin promoter by low-potency PARP inhibitors required an intact C1 element. **A:** Human insulin promoter sequences from  $-327$  to  $+30$  bp showing the location of known regulatory elements and some identified transcription factors. **B:** INS-1 cells were transfected with INS( $-230$ )CAT reporter genes containing mutated C1 [INS( $-230$ mC1)CAT]; A3 and A1 [INS( $-230$ mA3A1)CAT]; or C1, A3, and A1 [INS( $-230$ mC1A3A1)CAT] elements. Cells were incubated for 48 h in 16.7 mmol/l glucose (Glu) and nicotinamide (NAM) or 3-aminobenzamide (3-AB). \* $P < 0.05$ , wild-type versus mutant reporter genes for respective low-potency PARP inhibitors ( $n = 4$ ).

this purpose, INS-1 cells were pretreated with PARP inhibitors for 1.5 h and then incubated for 1 h with 1 mmol/l  $H_2O_2$ , after which cleavage of caspase 9 was determined by Western blot. Treatment of cells with  $H_2O_2$  increased the appearance of 38-kDa fragment of caspase 9, and this was blocked by all PARP inhibitors tested (Fig. 2B). PARP inhibitors also blocked  $H_2O_2$ -induced activation of caspase 3 activity (data not shown). These results show that PARP inhibitors are functional in INS-1 cells and suggest that attenuation of glucose suppression of insulin promoter activity is independent of inhibition of PARP-1 enzymatic activity.

**Antioxidants marginally attenuated glucose-mediated suppression of insulin promoter activity.** Nicotinamide and 3-aminobenzamide have also been reported to function as antioxidants (29,30). Antioxidants, including quercetin (10  $\mu$ mol/l),  $\alpha$ -lipoic acid (1 mmol/l), or *N*-acetylcysteine (NAC; 10 mmol/l), were thus tested for their ability to attenuate glucose suppression of insulin promoter activity. As shown above, treatment of cells with PD128763 increased ( $\sim 10$ -fold) insulin promoter activity suppressed by elevated glucose (Fig. 3A). In contrast, quercetin,  $\alpha$ -lipoic acid, or NAC only increased insulin promoter activity from 1.5- to 2.5-fold. Next, the effectiveness of the compounds were tested for their ability to block  $H_2O_2$ -induced oxidative stress in INS-1 cells. Cells were preloaded with a fluorescent indicator ( $H_2DCF$ ) of ROS, incubated for 1.5 h with PARP inhibitors or antioxidants and then treated with 1 mmol/l  $H_2O_2$  to induce

oxidative stress. In this assay, intracellular ROS converts nonfluorescent  $H_2DCF$  to fluorescent DCF. Treatment of cells with  $H_2O_2$  led to a 3.7-fold increase ( $n = 4$ ,  $P < 0.001$ ) in DCF fluorescence, indicating a significant increase in ROS generation (Fig. 3B). The increase in  $H_2O_2$ -induced ROS production was significantly inhibited by NAC or quercetin. Nicotinamide, 3-aminobenzamide, PD128763, and  $\alpha$ -lipoic acid had little effect on inhibition of  $H_2O_2$ -induced ROS production. These results suggest that attenuation of glucose suppression of insulin promoter activity by low-potency PARP inhibitors is independent of a generalized antioxidant effect.

**The C1 element participates in activation of insulin promoter activity by low-potency PARP inhibitors.** Several key regulatory elements have been identified in the human insulin gene promoter, including C1, A1 and A3, and E1 (rev. in 31), which serve as binding sites for PDX-1 (32), MafA (33,34), and  $\beta$ -cell E-box trans-activator 2 (BETA2) (35), respectively (Fig. 4A). We have reported that glucose-mediated suppression of insulin promoter activity in INS-1 cells is associated with reduced binding of PDX-1 to the A1 and A3 elements and MafA to the C1 element (4,9). To test whether these elements are involved in low-potency PARP inhibitor regulation of insulin promoter activity, the A1, A3, and C1 elements were mutated in the context of the  $-230$  insulin promoter. Mutation of the A1 and A3 elements [INS( $-230$ mA3A1)CAT] had little effect on the ability of nicotinamide or 3-aminobenzamide to activate the  $-230$  insulin promoter (Fig. 4B). In contrast,



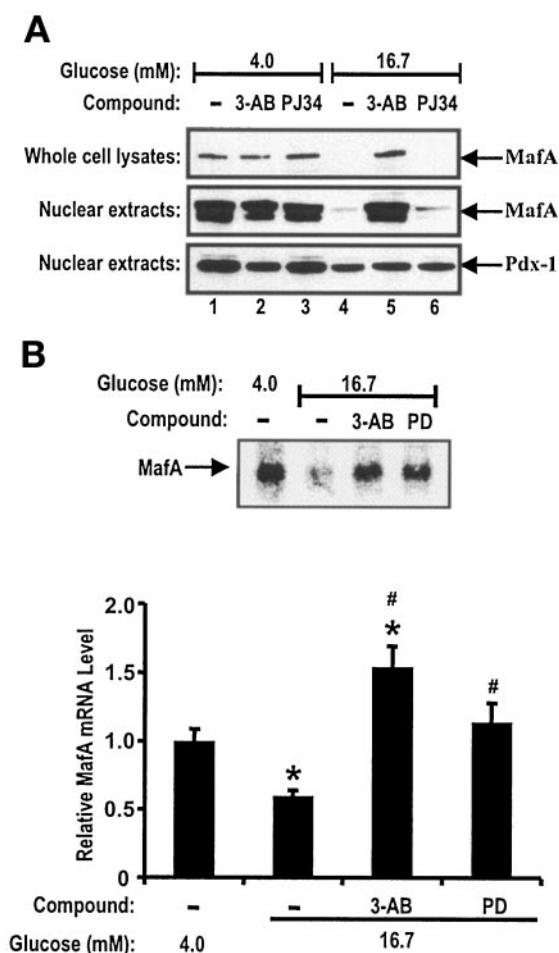
**FIG. 5.** Low-potency PARP inhibitors increased MafA binding activity. Nuclear extracts were isolated from INS-1 cells cultured for 48 h in 4 or 16.7 mmol/l glucose and vehicle, 3-aminobenzamide (3-AB), or PJ34. **A:** Electrophoretic mobility shift assay of MafA binding to the A2C1 element. Anti-MafA antibodies were added to lanes 4 and 5. **B:** Electrophoretic mobility shift assay of PDX-1 binding to the A3 element. Anti-PDX-1 antibodies were added to lane 8 ( $n = 3$ ).

mutation of the C1 element [INS(-230mC1)CAT] led to a ~60% decrease in the ability of nicotinamide or 3-aminobenzamide to activate insulin promoter activity. Combined mutation of the A1, A3, and C1 elements [INS(-230mA3A1C1)CAT] did not further reduce low-potency PARP inhibitor activation of insulin promoter activity. The ability of PD128763 to induce insulin promoter activity also mapped to the C1 element (data not shown). Moreover, nicotinamide induced -230 insulin promoter activity in cells cultured in low glucose, and this mapped to the C1 element (data not shown). These findings suggest that the C1 element, but not A1 and A3, participates in low-potency PARP inhibitor activation of insulin promoter activity, implicating a potential role for MafA.

**Low-potency PARP inhibitors increase MafA binding activity to the C1 element.** Electrophoretic mobility shift assays were performed to determine whether low-potency PARP inhibitors affected MafA binding to the C1 element. Treatment of cells with 16.7 mmol/l glucose led to a large reduction in C1 element binding activity (Fig. 5A, lanes 2, 3, 7, and 8). Anti-MafA antibodies were able to supershift the C1 element binding complex, indicating that these complexes contain MafA (Fig. 5A, lanes 4 and 5). Treatment of cells with 3-aminobenzamide, but not PJ34, increased C1 element binding activity in nuclear extracts derived from cells cultured in 16.7 mmol/l glucose (Fig. 5A, lanes 10 and 12). PDX-1 binding to the A3 element was reduced in nuclear extracts derived from INS-1 cells cultured in 16.7 mmol/l glucose (Fig. 5B). In contrast to MafA binding, 3-aminobenzamide treatment did not increase PDX-1 binding activity. Treatment of cells with nicotinamide or PD128763 also led to the restoration of C1 element binding activity (data not shown). These data show that low-potency PARP inhibitors can specifically increase MafA binding activity to the C1 element.

**Low-potency PARP inhibitors increase MafA protein and mRNA levels.** Treatment of INS-1 cells for 48 h with 16.7 mmol/l glucose led to a large reduction in MafA protein levels in whole-cell lysates and nuclear extracts (Fig. 6A). Elevated glucose (16.7 mmol/l) also led to a 41% reduction ( $n = 4$ ,  $P < 0.005$ ) in MafA mRNA levels (Fig. 6B). The addition of 3-aminobenzamide or PD128763, but not PJ34, led to a marked increase in MafA protein and mRNA levels (Fig. 6B and data not shown). In contrast to MafA, PDX-1 protein levels were not affected by treatment with either 3-aminobenzamide, PD128763, or PJ34.

**Induction of MafA mRNA by low-potency PARP inhibitors precedes increases in MafA protein.** A time course analysis was performed to determine the relationship between increased MafA mRNA and protein levels. In this series of experiments, INS-1 cells were switched from medium containing 11.1 mmol/l glucose to medium containing 4, 16.7, or 16.7 mmol/l glucose plus 3-aminobenzamide. MafA mRNA and protein levels were measured after 4, 8, 12, and 24 h. Insulin mRNA levels and promoter activity are suppressed in INS-1 cells cultured in 11.1 mmol/l glucose (4,9). The transition from 11.1 to 4 mmol/l glucose yielded up to a 1.5-fold increase ( $n = 4$ ,  $P < 0.05$ ) in MafA mRNA levels that were detectable within 12–24 h (Fig. 7A and B). Over this same time period, MafA protein levels were not significantly changed. Transition from 11.1 to 16.7 mmol/l glucose led to a significant reduction in MafA protein levels after 24 h, but this occurred with only a modest reduction in MafA mRNA. The addition of 3-aminobenzamide to cells cultured in 16.7 mmol/l glucose induced MafA mRNA within 4 h, and this preceded an increase in MafA protein levels. Treatment of cells with 3-aminobenzamide led to significant increases in MafA protein levels within 8 h. The addition of 3-aminobenzamide to cells cultured in 4 mmol/l glucose also led to a



**FIG. 6.** Low-potency PARP inhibitors increased MafA protein and mRNA levels. **A:** MafA or PDX-1 protein levels in whole-cell lysates or nuclear extracts isolated from INS-1 cells treated for 48 h with 4 or 16.7 mmol/l glucose and vehicle, 3-aminobenzamide, or PJ34. Representative Western blots are shown ( $n = 3$ ). **B, upper panel:** MafA mRNA expression in INS-1 cells cultured in 4 or 16.7 mmol/l glucose and vehicle, 3-aminobenzamide (3-AB), or PD128763 (PD). **B, lower panel:** MafA mRNA relative to  $\beta$ -actin mRNA and normalized to 4 mmol/l glucose ( $n = 4$ ). \* $P < 0.05$ , 4 vs. 16.7 mmol/l glucose; # $P < 0.05$ , 16.7 mmol/l glucose vs. low-potency PARP inhibitors.

rapid induction in MafA protein (data not shown). These data indicate that low-potency PARP inhibitors rapidly increase MafA protein levels, in part, through increased MafA gene expression.

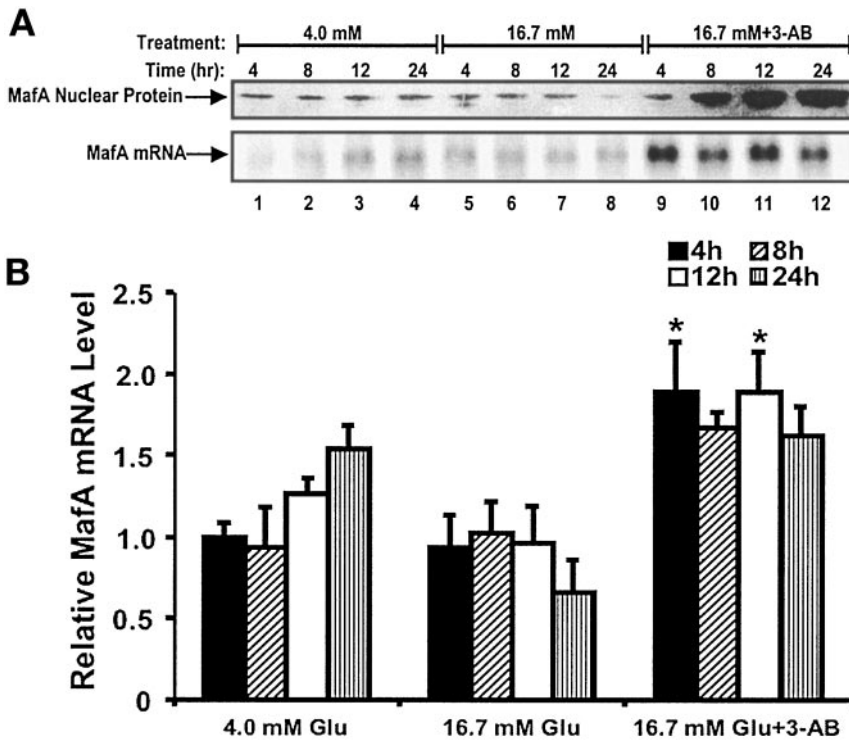
**Low-potency PARP inhibitors do not increase stability of MafA protein or mRNA.** To investigate whether low-potency PARP inhibitors increased MafA protein stability, INS-1 cells were first cultured for 12 h in 4 mmol/l glucose ( $t = 0$ ). The cells were then cultured for up to 24 h with the protein synthesis inhibitor cycloheximide (40  $\mu$ mol/l) in the absence or presence of 3-aminobenzamide. Cycloheximide treatment led to a time-dependent loss of MafA protein, and this was not affected by 3-aminobenzamide (Fig. 8A). These data suggest that low-potency PARP inhibitors do not induce MafA protein levels by increasing MafA protein stability. To test whether low-potency PARP inhibitors increased MafA mRNA stability, INS-1 cells were first cultured for 24 h in 16.7 mmol/l glucose and 3-aminobenzamide to induce MafA mRNA. The cells were then cultured for up to 24 h with the RNA synthesis inhibitor actinomycin D (1  $\mu$ g/ml) with or without 3-aminobenzamide. The addition of actinomycin D for

4 h led to a  $>95\%$  decrease in MafA mRNA, and this was unaffected by 3-aminobenzamide (Fig. 8B). This data suggest that low-potency PARP inhibitors do not induce MafA mRNA stability.

## DISCUSSION

Loss of insulin gene expression in  $\beta$ -cell lines exposed to elevated glucose results from reduced insulin promoter activity that is associated with reduced binding of PDX-1 and MafA (3,4,7,10). Studies presented herein show that low-potency PARP inhibitors can restore insulin promoter activity suppressed by elevated glucose, in part, by increasing MafA expression. Consistent with this observation, mutation of the C1 element, which serves as a MafA binding site, decreased low-potency PARP inhibitor activation of the  $-230$  human insulin promoter. Mutation of the C1 element, however, did not completely abolish the ability of low-potency PARP inhibitors to activate the  $-230$  promoter, suggesting the involvement of other regulatory elements. PDX-1 is unlikely to be involved because mutation of the A1 and A3 elements had no effect on low-potency PARP inhibitor activation of promoter activity, and low-potency PARP inhibitors did not affect PDX-1 protein levels or binding activity. Moreover, overexpression of PDX-1 in INS-1 cells does not restore insulin promoter activity reduced by elevated glucose (9). A MafA response element has been located on the human insulin promoter from sequences  $-186$  to  $-174$  bp (36) and overlaps with the CRE/CAAT element. Consistent with this sequence functioning as a MafA response element, site-directed mutation of the CRE/CAAT element led to a  $\sim 30\%$  reduction in low-potency PARP inhibitor induction of insulin promoter activity (data not shown). Overall, these data support the conclusion that low-potency PARP inhibitors can increase  $-230$  insulin promoter activity in INS-1 cells cultured in elevated glucose primarily by modulating MafA expression. Further studies are necessary to determine the role of MafA for induction of insulin mRNA levels and promoter activity by low-potency PARP inhibitors under low-glucose conditions.

Nicotinamide and 3-aminobenzamide can function as antioxidants (29,30), suggesting that the low-potency PARP inhibitors may attenuate glucose-mediated suppression of insulin promoter activity and MafA protein levels via reducing oxidative stress. This is an attractive possibility because  $\beta$ -cells have low intrinsic antioxidant defense mechanisms, making them particularly susceptible to damage by oxidative stress (14). Oxidative stress as a mechanism for suppression of insulin promoter activity is supported by the ability of antioxidants to attenuate  $H_2O_2$ - or D-ribose-mediated loss of insulin gene expression and PDX-1 binding activity (6,12,13). Moreover, NAC prevents loss of insulin gene expression and MafA protein levels in HIT-T15 cells chronically passaged (up to 1 year) in elevated glucose (11). For these reasons, antioxidants, including NAC, quercetin, and  $\alpha$ -lipoic acid, were tested for the ability to attenuate glucose suppression of insulin promoter activity. Compared with low-potency PARP inhibitors, antioxidants were relatively ineffective at stimulating insulin promoter activity. NAC and quercetin were, however, effective at preventing  $H_2O_2$ -induced ROS production in INS-1 cells. In contrast, low-potency PARP inhibitors did not efficiently block  $H_2O_2$ -induced intracellular ROS. These results indicate that low-potency



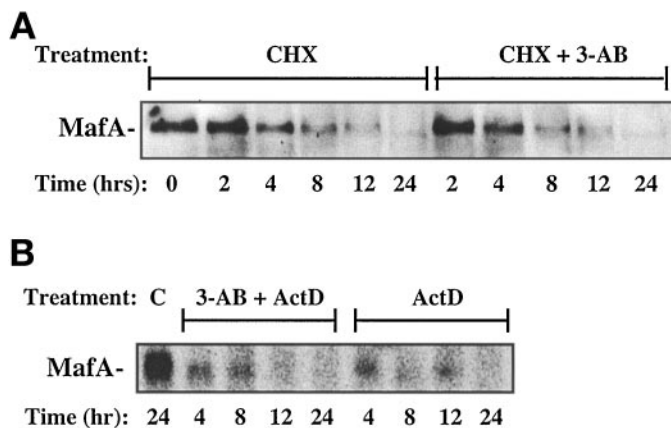
**FIG. 7.** Induction of MafA mRNA by low-potency PARP inhibitors precedes increases in MafA protein. **A:** Time course for induction of MafA mRNA ( $n = 4$ ) and protein ( $n = 3$ ) by 3-aminobenzamide (3-AB). Representative Western and Northern blots are shown. **B:** MafA mRNA relative to  $\beta$ -actin mRNA and normalized to 4 mmol/l glucose (Glu). \* $P < 0.05$ , 4 mmol/l glucose vs. 3-aminobenzamide for the given time point.

PARP inhibitors attenuated glucose suppression of insulin promoter activity and MafA levels through pathways independent of an antioxidant mechanism. These results are in opposition to that of Harmon et al. (11), who reported that NAC prevents loss of insulin gene expression and MafA protein in chronically passaged HIT-T15 cells. The inconsistency between these studies are most likely caused by differences in cell lines used and length of culture. For example, loss of insulin gene expression and MafA require months for HIT-T15 cells, whereas similar changes occur within 24 h for INS-1 cells. More important, the HIT-T15 cell studies involved a vast number of cell doublings, leaving the possibility that NAC is selecting for a MafA-containing population.

NAC has been used to select for rapidly growing cell populations from human islets that retain the capacity to express insulin (17). Selection of INS-1 cells within 24–48 h is unlikely to account for the ability of low-potency PARP inhibitors to restore loss of insulin promoter activity and MafA.

Prolonged exposure of various tissues to elevated glucose causes oxidative stress (rev. in 14) and can activate PARP-1 (37,38). This suggests that low-potency PARP inhibitors might increase insulin promoter activity and MafA levels by inhibiting PARP-1 activity. Because PARP-1 is the most abundant form of PARP in mammalian cells and influences transcription factor and coactivator activity (39,40), two potent PARP-1 inhibitors were tested for their ability to modulate insulin promoter activity and MafA. Both PJ34 and INO-1001 at concentrations that inhibit PARP-1 in cultured cells (37,38) were ineffective at attenuating glucose suppression of insulin promoter activity and MafA levels. Higher concentrations of potent PARP-1 inhibitors were also tested, but they were cytotoxic (data not shown). PJ34 and INO-1001, however, blocked  $H_2O_2$ -induced apoptosis in INS-1 cells, as demonstrated by prevention of cleavage of caspase 9. These data indicate that low-potency PARP inhibitors do not regulate insulin promoter activity or MafA protein levels through a mechanism involving PARP-1. There are, however, at least seven forms of PARPs in mammalian cells that contain a highly conserved  $\beta$ -NAD<sup>+</sup> binding site targeted by low-potency PARP inhibitors (40).

Differences in the ability of low-potency PARP inhibitors and potent PARP-1 inhibitors to induce insulin promoter activity are likely caused by their chemical structures and concentrations used. Most PARP inhibitors are monocyclic carboxamides (nicotinamide and 3-aminobenzamide), bicyclic lactams (PD128763), or polycyclic lactams (PJ34) (41). The amide group of nicotinamide or 3-aminobenzamide can rotate relative to the plane of the aromatic ring, making them less potent for PARP inhibi-



**FIG. 8.** Low-potency PARP inhibitors do not stabilize MafA protein or mRNA levels. **A:** Time course for MafA protein degradation in INS-1 cells cultured in 4 mmol/l glucose and 40  $\mu$ mol/l cycloheximide (CHX) in the absence or presence of 3-aminobenzamide (3-AB; 10 mmol/l). Representative Western blot is shown ( $n = 2$ ). **B:** Time course for MafA mRNA degradation in INS-1 cell cultured in 16.7 mmol/l glucose and 1  $\mu$ g/ml actinomycin D (ActD) with or without 3-aminobenzamide. Representative Northern blot is shown ( $n = 3$ ). C, control cells cultured for 24 h in 16.7 mmol/l glucose plus 3-aminobenzamide.

tion. Constraining mono-aryl carboxamides into bicyclic lactams increases potency. Three-ring structures further increase the potency, thus making PJ34 more potent than PD128763. Because low-potency PARP inhibitors are less potent, higher concentrations are necessary to inhibit PARP activity, increasing the likelihood that other  $\beta$ -NAD<sup>+</sup> binding proteins are nonselectively affected. High concentrations of low-potency PARP inhibitors inhibit other  $\beta$ -NAD<sup>+</sup> binding proteins, including mono(ADP-ribose) transferases and NADases (42). Nicotinamide can increase gene expression by interfering with the  $\beta$ -NAD<sup>+</sup> binding site of Sir2 (silent information regulatory 2) family of histone/protein deacetylases (43) or the transcriptional corepressor CtBP (COOH-terminal binding protein) (44). This raises the possibility that low-potency PARP inhibitors modulate  $\beta$ -cell gene expression by effecting transcriptional corepressors. Indeed, FoxO1 (forkhead box O1) can protect  $\beta$ -cells against oxidative stress by complexing with promyelocytic leukemia protein Pml and Sirt1, the mammalian homologue of Sir2, to activate BETA2 and MafA (45).

Although the target of low-potency PARP inhibitors is unidentified, the mechanism whereby low-potency PARP inhibitors increase MafA protein levels in INS-1 cells cultured in elevated glucose involves increased expression of MafA mRNA. This is best illustrated by the ability of 3-aminobenzamide to induce MafA mRNA levels within 4 h, followed by a subsequent increase in MafA protein levels at 8 h (Fig. 7). This rapid induction of MafA mRNA levels could be caused either by increased MafA gene transcription and/or decreased MafA mRNA degradation. 3-aminobenzamide, however, did not delay MafA mRNA degradation in cells treated with actinomycin D. These results strongly suggest that low-potency PARP inhibitors induce MafA mRNA expression through stimulation of MafA gene transcription. There is precedence for MafA mRNA levels being regulated by glucose at the level of transcription in MIN6 and  $\beta$ -TC6 cells (33). Moreover, oxidative stressors can rapidly induce members of the MafA transcription factor family, such as MafG (46). It remains to be determined whether the MafA gene promoter contains regulatory regions responsive to low-potency PARP inhibitors or changes in cellular  $\beta$ -NAD<sup>+</sup> levels.

In HIT-T15 cells, MafA protein degradation involves lactacystin-sensitive 20S proteasome activity (11). Oxidative stress can lead to proteasome activation, and this is dependent on poly(ADP-ribosyl)ation of proteasomal proteins (47); thus, it is possible that low-potency PARP inhibitors may stabilize MafA protein via inhibition of proteasome activity. This is unlikely, however, because 3-aminobenzamide did not affect MafA protein degradation in INS-1 cells treated with the protein synthesis inhibitor cycloheximide. Moreover, lactacystin, which selectively inhibits 20S proteasome activities, and MG132 (carbonyloxyl-leuciny-leuciny-leucinal-H), which selectively inhibits 20S and 26S proteasome activities, did not increase MafA protein levels in INS-1 cells cultured in elevated glucose (data not shown). These data suggest that low-potency PARP inhibitors do not induce MafA protein levels by increasing MafA protein stability.

Overall, our results demonstrate that nicotinamide and other low-potency PARP inhibitors increase insulin promoter activity in INS-1  $\beta$ -cells by increasing MafA gene expression. The implication of this study is broad

because many protocols for differentiating fetal islets, pancreatic precursor/stem cells, and embryonic stem cells into insulin-producing cells use millimolar concentrations of nicotinamide. PD128763 at a concentration as low as 100  $\mu$ mol/l was able to increase insulin promoter activity (data not shown). Assuming that the mechanism(s) whereby nicotinamide increases insulin biosynthesis in fetal islets, pancreatic precursor cells, and embryonic stem cells is similar to that for INS-1 cells, it is likely that more specific compounds can be designed to target these processes.

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