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Maintenance of Redox State and Pancreatic Beta-Cell Function: Role of Leptin and Adiponectin

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ABSTRACT

Whereas oxidative stress is linked to cellular damage, reactive oxygen species (ROS) are also believed to be involved in the propagation of signaling pathways. Studies on the role of ROS in pancreatic beta-cell physiology, in contrast to pathophysiology, have not yet been reported. In this study we investigate the importance of maintaining cellular redox state on pancreatic beta-cell function and viability, and the effects of leptin and adiponectin on this balance. Experiments were conducted on RINm and MIN6 pancreatic beta-cells. Leptin (1–100 ng/ml) and adiponectin (1–100 nM) increased ROS accumulation, as was determined by DCFDA fluorescence. Using specific inhibitors, we found that the increase in ROS levels was mediated by NADPH oxidase (Nox), but not by AMP kinase (AMPK) or phosphatidyl inositol 3 kinase (PI3K). Leptin and adiponectin increased beta-cell number as detected by the XTT method, but did not affect apoptosis, indicating that the increased cell number results from increased proliferation. The adipokines-induced increase in viability is ROS dependent as this effect was abolished by N-acetyl-L-cysteine (NAC) or PEG-catalase. In addition, insulin secretion was found to be regulated by alterations in redox state, but not by adipokines. Finally, the effects of the various treatments on activity and mRNA expression of several antioxidant enzymes were determined. Both leptin and adiponectin reduced mRNA levels of superoxide dismutase (SOD)1. Adiponectin also decreased SOD activity and increased catalase and glutathione peroxidase (GPx) activities in the presence of H_2O_2. The results of this study show that leptin and adiponectin, by inducing a physiological increase in ROS levels, may be positive regulators of beta-cell mass. J. Cell. Biochem. 113: 1966–1976, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: LEPTIN; ADIPONECTIN; REACTIVE OXYGEN SPECIES; PANCREATIC BETA-CELL

Reactive oxygen species (ROS) are by-products of normal metabolic activity, and are also produced as a consequence of several environmental factors [Zadak et al., 2009]. O_2 is capable of giving rise to reactive excited states such as free radicals and derivatives, including superoxide radical (O_2\cdot−), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Both O_2\cdot− and $OH^{\cdot}$ are extremely reactive and can interact with proteins, lipids, and nucleic acids leading to extensive molecular damage [Auten and Davis, 2009]. Accumulation of ROS as a result of increased production or reduced antioxidants leads to oxidative stress, a common denominator in many diseases, such as cancer, neurodegenerative diseases, and presumably also diabetes [Valko et al., 2007].

Whereas it is generally believed that development of insulin resistance and pancreatic beta-cell death are linked to the presence of oxidative stress [Evans et al., 2005; Fridlyand and Philipson, 2005; Lenzen, 2008; Bashan et al., 2009], several studies found that administration of antioxidants is ineffective for prevention of diabetes mellitus [Song et al., 2009] and may even accelerate the failure of beta-cells leading to the development of type 1 or type 2 diabetes [Stranges et al., 2007; Ristow et al., 2009]. These disappointing results could be either because excessive antioxidants

Abbreviations used: AMPK, AMP kinase; CM-H_2DCFDA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; DPI, diphenylene-iodonium; GPx, glutathione peroxidase; GSIS, glucose stimulated insulin secretion; KRBH, Krebs' Ringer bicarbonate HEPES; NAC, N-acetyl-L-cysteine; Nox, NADPH oxidase; PI3K, phosphatidyl inositol 3 kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance.

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might be converted to pro-oxidants, or because various ROS may have differential effects on physiological and pathological processes. In this regard, it has recently become apparent that 'non-damaging', strictly regulated levels of ROS are not always harmful byproducts. ROS, especially H_2O_2 and O_2\cdot− are highly regulated metabolites which are involved in several important physiological functions in cells [Giorgio et al., 2007]. ROS act as intracellular signaling molecules mediating gene expression, cell proliferation, differentiation, and viability [Droge, 2002]. Transient bursts of ROS lead to a temporary shift of the intracellular redox state, affecting signaling pathways such as insulin signaling, MAPK, and JNK [Goldstein et al., 2005; Forman et al., 2010]. Thus, tight regulation of the redox potential of cells is crucial for maintenance of normal cell function. While the deleterious effects of increased ROS levels on pancreatic beta-cell function are well documented, the importance of well-regulated ROS production on insulin secretion and beta-cell proliferation and survival has not yet been reported.

Several hormones are known to regulate beta-cell function, among which are leptin and adiponectin, secreted by adipose tissue, whose function is known to be crucial for protection against the development of metabolic syndrome and diabetes [Weyer et al., 2001; Oral et al., 2002]. Leptin and adiponectin improve insulin sensitivity of peripheral target tissues [Stefan et al., 2002; Paz-Filho et al., 2008], and play a role in the regulation of pancreatic beta-cell function [Rakatzi et al., 2004; Covey et al., 2006]. Several studies show involvement of leptin and adiponectin in the regulation of redox state. Anti-oxidative effects of adiponectin prevented steatohepatitis in mice [Fukushima et al., 2009] and LDL oxidation in vascular endothelial cells [Ouedraogo et al., 2006]. Leptin protects hepatoma cell line against ethanol-induced oxidative stress [Balasubramaniyan et al., 2007], but increase ROS production in monocytes [Sanchez-Pozo et al., 2003] and keratinocytes [Savini et al., 2003]. These various and potentially conflicting results indicate the need to clarify the role of leptin and adiponectin on redox regulation and their involvement in protection against oxidative stress, particularly in pancreatic beta-cells. Accordingly, in this study, we have investigated the importance of redox regulation on pancreatic beta-cell function, and the effects of leptin and adiponectin on regulation of cellular oxidative state.

MATERIALS AND METHODS

MATERIALS

Reagents and media for cell cultures, recombinant rat leptin and globular adiponectin were obtained from Biological Industries (Beith Haemek, Israel). ELISA detection kit for insulin was purchased from Mercodia. N-acetyl-L-cysteine (NAC) was obtained from Merck. PEG-catalase was obtained from Sigma, diphenylene-iodonium (DPI), compound C and LY-294002 were purchased from Calbiochem.

CELL CULTURE

Rat RINm insulinoma cells were grown in RPMI 1640 medium, containing 10% fetal calf serum (FCS), 2 mM glutamine, 1% ampicilin, 11 mM glucose, 10 mM Hepes buffer, and 1 mM sodium pyruvate. Mouse MIN6 insulinoma cells were grown in DMEM medium, containing 10% FCS, 25 mM glucose, 2 mM glutamine, 1% ampicilin, 64 μM beta-mercaptoethanol. Cells were grown at 37°C, in a humidified atmosphere containing 5% CO_2.

INSULIN SECRETION STUDIES

MIN6 cells were cultured at a concentration of 10^5 cells/ml. After 48 h, cells were treated with leptin, adiponectin, NAC, or H_2O_2 for 40 min. Cells were preincubated in KRBH buffer containing 25 mM $NaHCO_3$, 115 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, 0.1% BSA for 30 min and the supernatant was collected. The cells were then incubated in KRBH buffer containing 15 mM glucose for an additional 30 min. The supernatant was collected, and insulin concentration at the basal state and after induction was measured using an insulin immunoassay kit (Mercodia) according to the manufacturer's instructions.

DETERMINATION OF CELL VIABILITY

Cell viability was measured by XTT Cell proliferation kit (Biological Industries) or cellTiter blue Cell Proliferation Assay (CTB, Promega). For the XTT method, RINm and MIN6 cells were subcultured at a concentration of 10^5 cells/ml. Twenty four hours after seeding, cells were treated with leptin or adiponectin, with or without the addition of NAC or PEG-catalase in a growth media containing 2% FCS. After an additional 24 h, cells were washed and incubated with XTT for 4 h according to the manufacturer's instructions. Optical density (OD) was measured using a Tecan Infinite F200 fluorescence microplate reader (Tecan, Salzburg, Austria) at a test wavelength of 492 nm and a reference wavelength of 690 nm. For the CTB proliferation assay, MIN6 cells were seeded at a density of 10^5 cells/ml. Twenty four hours after seeding, cells were treated with leptin or adiponectin, in growth medium containing 2% FCS. After an additional 24 h, cells were washed and incubated with CTB reagent (20 μl/well). The cells were incubated at 37°C for a further 2–3 h, and then fluorescence was measured at 560/590 nm.

ANALYSIS OF mRNA EXPRESSION BY PCR REACTIONS

Total RNA was extracted from RINm cells using PerfectPure tissue RNA Kit (5 PRIME, Gaithersburg). 2.5 ng of total RNA were reverse transcribed by oligo dT priming (Stratascript, Stratagene) according to the manufacturer's instructions. Real-time PCR amplification reactions were performed using SYBRGreen Master mix (ROVA-LAB), by the MxPro QPCR instrument (Stratagene).

Primer sequences and their respective PCR fragment length were as follows: Preproinsulin (160 bp): Forward 5′-tcaaacagcaccttt-3′, reverse 5′-agtgccaaggtctga-3′. Superoxide dismutase (SOD)1 (204 bp): Forward 5′-tgcagggcgtcattcactt-3′, reverse 5′-caacatg-cctctcttcatcc-3′. SOD2 (197 bp): Forward 5′-cacattaacgcgcagat-3′, reverse 5′-ctcaggtttgtccagaaa-3′. Catalase (220 bp): Forward 5′-agcagtggaaggagcagc-3′, reverse 5′-tcaaagtatccaaaagcacc-3′. Gluta-thione peroxidase (GPx) (160 bp): Forward 5′-cat gaccgaccccaag-taca-3′, reverse 5′-gggttgctaggctgcttgga-3′. Rat HPRT was used as housekeeping gene (130 bp): Forward 5′- aggccagactttgttggat-3′, reverse 5′-gcttttccactttcgctgat-3′.

DETECTION OF INTRACELLULAR ROS ACCUMULATION

Cellular oxidative stress was measured using 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Sigma). RINm and MIN6 cells were seeded at a density of 2.0×10^5 cells/ml. Two days after seeding cells were treated with 10 μM CM-H_2DCFDA for 30 min in the dark. Following treatment, cells were incubated with the appropriate growth medium for 15 min to recovery. Background fluorescence was determined using microplate reader set at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Finally, cells were treated with leptin or adiponectin for 40 min. Fluorescence was detected, and for each well the value was corrected for intracellular protein and background fluorescence.

DETECTION OF INTRACELLULAR H_2O_2 PRODUCTION

H_2O_2 generation was detected using Amplex Red reagent (Molecular Probes). RINm and MIN6 cells were seeded at a density of 2.0×10^5 cells/ml. Two days after seeding cells were treated for 40 min with leptin or adiponectin, washed twice with cold PBS, and scraped in the presence of Sodium-phosphate buffer (0.25M, pH 7.4). Lysates were centrifuged at 2,500*g*, at 4°C for 10 min. H_2O_2 was detected immediately using Amplex Red reagent according to the manufacturer's instructions.

LIPID PEROXIDATION ANALYSIS

Lipid peroxidation was quantified using the thiobarbituric acid reactive substance (TBARS) assay. RINm and MIN6 cells were seeded at a density of 2.0×10^5 cells/ml. Two days after seeding cells were treated for additional 24 h with leptin, adiponectin, or with H_2O_2 (70 μM) as a positive control. Cells (adherent and detached cells) were collected in cold PBS and centrifuged at 3,000 rpm for 10 min. The pellet was resuspended and lysed in Sodium-phosphate buffer (0.25 M, pH 7.4). Lysates were centrifuged at 14,000 rpm, at 4°C for 10 min, and supernatant was collected. Two hundred microliter of cell lysates, 200 μl of SDS 8.1%, 1.5 ml of acetic acid (20%) pH 3.5, 1.5 ml TBA 1%, and 1 ml water were added in this order to a tube. The mixture was vortexed, and the reaction was carried out in a boiling water bath for 1 h. The mixture was allowed to cool, and centrifuged at 3,000 rpm for 15 min. OD was measured spectrophotometrically at 532 nm by a Tecan Infinite F200 microplate reader (Tecan, Salzburg, Austria). Values were calculated as nmol MDA/mg protein according to a calibration curve of 1,1,3,3-tetraethoxypropane.

MEASUREMENT OF ANTIOXIDANT ENZYMES ACTIVITY

RINm cells were cultured at a concentration of 3×10^5 cells/ml. After 72 h, cells were treated with leptin or adiponectin with or without the addition of H_2O_2 for 40 min. Activity of antioxidant enzymes was measured in cell lysates. GPx activity was measured using GPx assay kit, SOD activity was measured using SOD Assay kit and Catalase activity was measured using Catalase assay kit (all purchased from Cayman chemical) according to manufacturer's instructions.

INDUCTION AND DETECTION OF APOPTOSIS

RINm cells were seeded at a concentration of 3×10^5 cells/ml. Apoptosis was induced by the different stimuli 48 h after seeding in serum-free medium, containing 0.5% BSA. Apoptosis was induced either by H_2O_2 (70 μM), palmitic acid (250 μM, as described by Rakatzi et al. [2004], or by cytokine mix. Cytokine mix was composed of: IL-1ß (50 U/ml); rat IFNγ (100 U/ml); rat TNFα (1,000 U/ml), (All were purchased from Peprotech). In both cases, cells were treated with or without the addition of leptin or adiponectin.

Apoptosis was detected by measuring DNA fragmentation and caspase-3 activity. DNA fragmentation was determined by measuring mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates using a cell death detection ELISA kit (Roche), according to the manufacturer's instructions. Caspase-3 activity was measured using a caspase-3 colorimetric assay kit (MBL) according to the manufacturer's instructions. Results were normalized to protein concentration in each well, which was measured using Bradford reagent.

STATISTICAL ANALYSIS

Values are presented as means ± SEM. Statistical differences between the treatments and controls were tested by unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's posthoc testing, when appropriate. Analysis was performed using the GraphPad Prism 5.0 software. A difference of $P < 0.05$ or less in the mean values was considered statistically significant.

RESULTS

H_2O_2 AT LOW CONCENTRATION INCREASES PANCREATIC BETA-CELL'S VIABILITY

Dose-dependent curves of effects of H_2O_2 and NAC on the viability of RINm pancreatic beta-cells are shown in Figure 1. High concentrations of both H_2O_2 (70 μM) and NAC (10 mM) were found to reduce beta-cell viability (viability of 22 and 58% respectively, compared to control). These results indicate that disturbance of the redox balance, either to the oxidative or to the reductive state may be harmful. However, whereas low concentrations of NAC did not affect cell viability, a low concentration of H_2O_2 (4 μM) was not only non-toxic, it actually increased the viability compared to control untreated cells. Treatment of cells with the damaging concentration of H_2O_2 (70 μM) combined with different concentrations of NAC (0.15–10 mM), neutralized the oxidative effect of H_2O_2 and reduced cell mortality. Combined treatment of H_2O_2 with 5 mM NAC even increased the viability of cells compared to controls. However, over-neutralization of H_2O_2, as observed following treatment with higher concentration of NAC (10 mM) abrogated this effect, indicating that there is an optimal redox balance that favors cell proliferation (Fig. 1C). In another series of experiments, cells were treated with 10 mM NAC, combined with different concentrations of H_2O_2 (Fig. 1D). An increase in viability compared to control was detected in cells treated with 10 mM NAC and H_2O_2 at 17.5 and 35 μM. This effect was not obtained in cells treated with 10 mM NAC combined with higher (70 μM) or lower (2–8 μM) H_2O_2 concentrations.

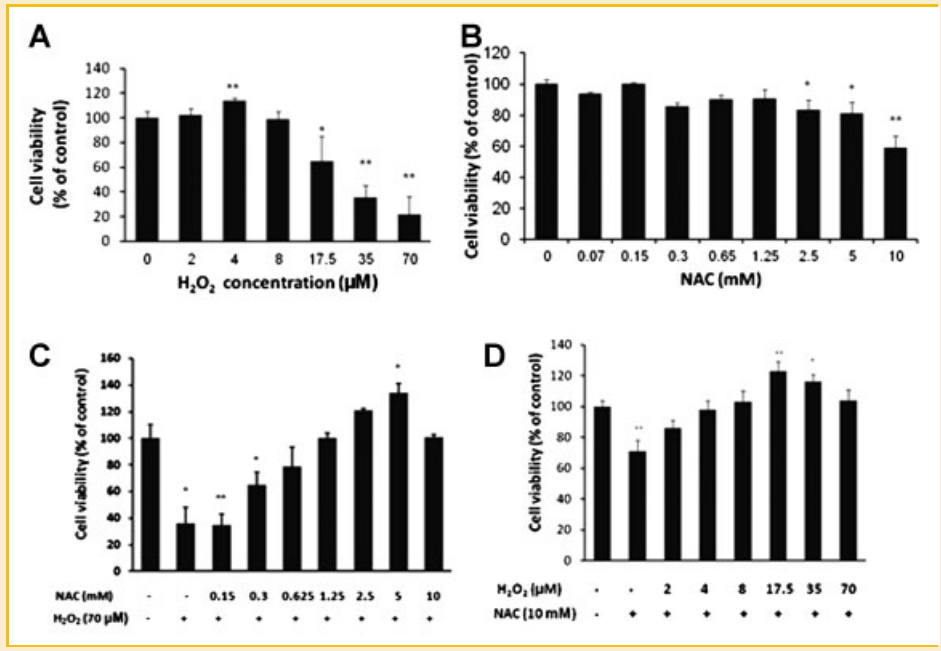

Fig. 1. H_2O_2 at low concentration increases and in high concentration decreases viability of pancreatic beta-cells. RINm cells were plated at a density of 10^5 cells/ml. Twenty four hours after plating cells were treated with (A) different concentrations of H_2O_2, (B) NAC, (C) combination of H_2O_2 (70 μM) and different concentrations of NAC (0.15–10 mM), or (D) combination of NAC (10 mM) and different concentrations of H_2O_2 (2–70 μM) for 24 h. Viability was measured 24 h after treatment using the XTT method, as described in Materials and Methods section. Each point represents the mean ± SEM of five independent experiments. $^*P < 0.05$, $^{**}P < 0.005$ by one-way ANOVA.

LEPTIN AND ADIPONECTIN INDUCE ROS GENERATION

Studies on the role of leptin and adiponectin in regulation of redox state in pancreatic beta-cells have not been reported. Accordingly, we next examined effects of these adipokines on ROS generation in the RINm and MIN6 cell lines. Dose response curves of ROS accumulation by adiponectin and leptin in RINm cells are presented in Figure 2A and B, respectively. On the base of these results, 10 nM adiponectin and 10 ng/ml of leptin were used for subsequent experiments. As shown in Figure 2C, both adipokines increased ROS generation in RINm and MIN6 cell line, the effect of adiponectin being considerably stronger than that of leptin. Thus, leptin increased ROS generation by 347% ($P < 0.0005$) in MIN6 cells and by 186% ($P < 0.005$) in RINm cells, whereas adiponectin increased ROS production by 841% ($P < 0.0005$) in MIN6 cells and by 411% ($P < 0.0005$) in RINm cells. This ROS accumulation was much lower than that which resulted from addition of H_2O_2 (1,297 and 4,029% in RINm and MIN6, respectively compared to control; data not shown). Pretreatment with the antioxidant NAC blocked ROS generation induced by the adipokines and H_2O_2 in both RINm (Fig. 2D) and MIN6 cells (data not shown). H_2O_2 levels (measured specifically by the Amplex red reagents) were increased following adiponectin treatment by 144 and 156%, respectively in RINm and MIN6 compared to control (Fig. 2E). The magnitude of the effect of adiponectin was much lower as determined by H_2O_2 levels specifically compared to total ROS levels. In addition, the increase in H_2O_2 in RINm cells following treatment with leptin was not statistically significant. These findings indicate that there are other ROS in addition to H_2O_2 generated by beta-cells in response to leptin and adiponectin. The possibility that the increase in oxidation of DCFDA may be attributed to an increase in reactive nitrogen species (RNS) appears to be negated by experiments in which we failed to observe an increase in RNS following treatment with leptin or adiponectin (RNS were measured using the Griess reaction, data not shown). The adipokines-induced increase in intracellular ROS was not followed by lipid peroxidation, a marker of oxidative cell damage (Fig. 2F), as was measured by the TBARS assay using RINm cells, indicating that the increase in ROS concentration does not reach pathological levels.

LEPTIN AND ADIPONECTIN REGULATE mRNA EXPRESSION AND ACTIVITY OF ANTIOXIDANT ENZYMES

In order to further examine the effects of leptin and adiponectin on the oxidative state of pancreatic beta-cells, we measured the activity and mRNA level of several major antioxidant enzymes in RINm cells (Fig. 3). SOD is an enzyme which converts the high-reactive radical O_2\cdot− to the less reactive radical H_2O_2. There are three isoforms of SOD: SOD1 is cytoplasmic, SOD2 is mitochondrial, and SOD3 is extracellular. In preliminary experiments, we found that SOD1 mRNA expression in beta-cells is 14.5 times higher than the expression of SOD2, and 2,732 times higher than that of SOD3, which was barely detected (data not shown). Accordingly we studied the effect of leptin and adiponectin on the expression of SOD1 and SOD2 but not SOD3.

As shown in Figure 3A, total activity (cytosolic and mitochondrial) of SOD was inhibited by H_2O_2 and adiponectin. Combined treatment of H_2O_2 and adiponectin had a greater inhibitory effect on

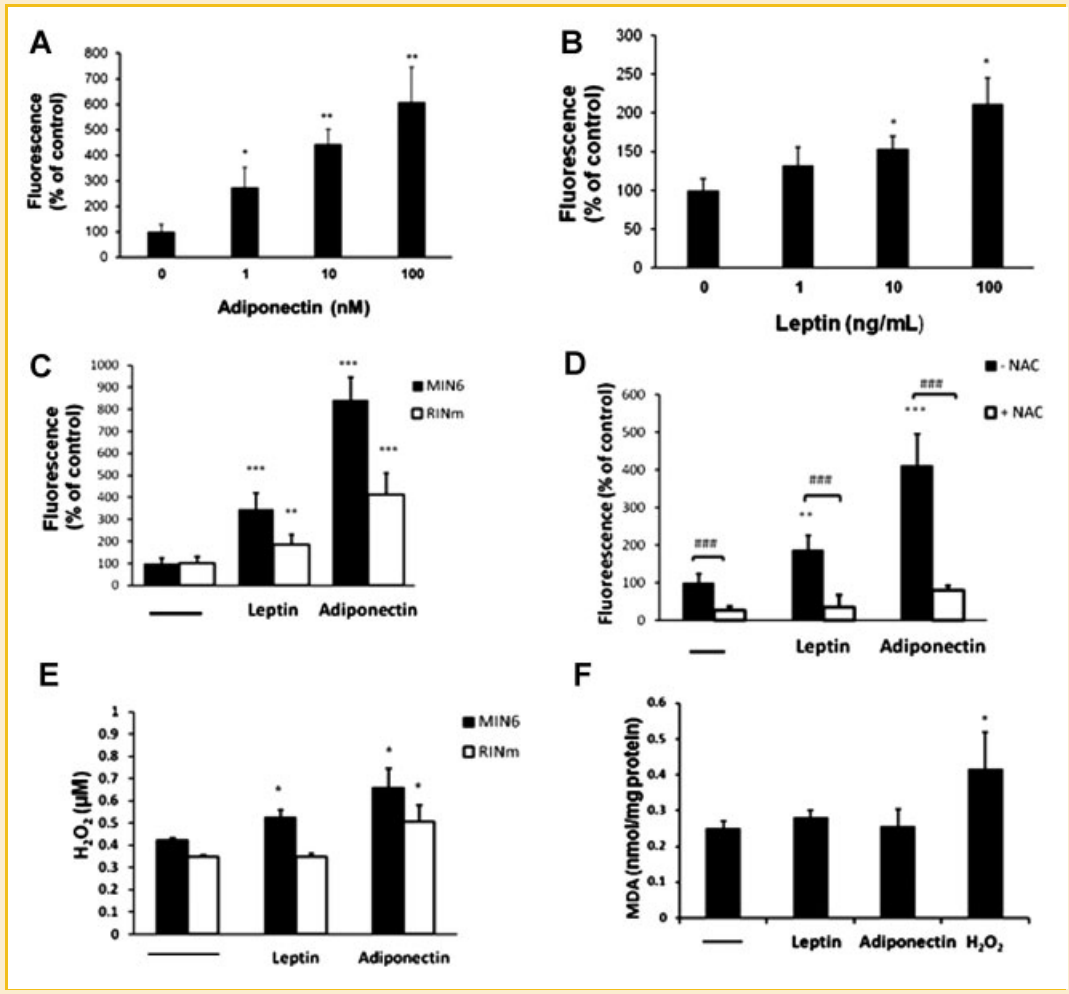

Fig. 2. Leptin and adiponectin induce ROS generation by pancreatic beta cells. RINm and MIN6 cells were plated at a density of 10^5 cells/ml. Twenty four hours after plating, cells were treated with leptin (1–100 ng/ml) or adiponectin (1–100 nM) for 40 min. ROS generation was measured as described in Material and Methods section. A, B: Dose response curves of ROS generation in RINm cells by adiponectin (A) and leptin (B). C: Leptin and adiponectin increase total ROS in RINm and MIN6 cell lines. Cells were treated with leptin (10 ng/ml) or adiponectin (10 nM) for 40 min. ROS generation was measured as described. D: NAC abrogates ROS generation in RINm cells. Cells were treated or not with NAC (10 mM) for 20 min before the addition of adipokines. E: Leptin and adiponectin increase H_2O_2 production. RINm and MIN6 cells were plated at a density of 10^5 cells/ml and treated as in Figure 2C. H_2O_2 was detected using Amplex red reagent, as described in Material and Methods section. F: Lipid peroxidation is not increased following treatment with leptin and adiponectin. RINm cells were plated at a density of 10^5 cells/ml. Twenty four hours after plating, cells were treated with leptin (10 ng/ml), adiponectin (10 nM), or H_2O_2 (70 μM) for 24 h. TBARS were measured as described in Material and Methods section. In all graphs, results are mean ± SEM of five independent experiments. ($^{**}P < 0.005$, $^{***}P < 0.0005$ compared to untreated cells, $^{\#\#\#}P < 0.0005$ compared to the appropriate treatment without NAC, by ANOVA.)

SOD activity than adiponectin alone. mRNA levels of SOD1 but not of SOD2 were also reduced by adiponectin (Fig. 3D,E). Thus, adiponectin i-nhibited both activity and mRNA expression of SOD1. Although H_2O_2 is less reactive than O_2\cdot−, excess intracellular H_2O_2 can attack molecules and induce oxidative damage as well. Thus, H_2O_2 is neutralized by the activity of catalase, which catalyses the reaction in which H_2O_2 is converted to H_2O and O_2, or by the activity of GPx, which couples the reduction of H_2O_2 to the oxidation of glutathione. As shown in Figure 3B,C, Catalase and GPx activities were not affected by either adiponectin or H_2O_2, but were increased following combined treatment of these agents. In addition, mRNA expression of GPx was increased by adiponectin (Fig. 3F). However, in spite of the stimulatory effect of adiponectin on catalase activity in the presence of H_2O_2, mRNA expression of catalase was reduced by adiponectin (Fig. 3G). These results indicate that under acute oxidative stress, adiponectin modifies the activity of the defensive antioxidant enzymes to a state of reduced production and increased disposal of H_2O_2.

In contrast, whereas leptin by itself did not affect SOD activity, it blocked the H_2O_2-dependent reduction in the activity of SOD (Fig. 3A) and reduced SOD1 mRNA (Fig. 3D). Catalase activity and its mRNA expression were not affected by leptin (Fig. 3B,F). Contrary to this, leptin decreased the activity of GPx in the presence of H_2O_2 and increased GPx mRNA expression (Fig. 3 C, G). Thus, interestingly, under acute oxidative stress, leptin increases the production and inhibits the disposal of H_2O_2 by modifying SOD and GPx activity.

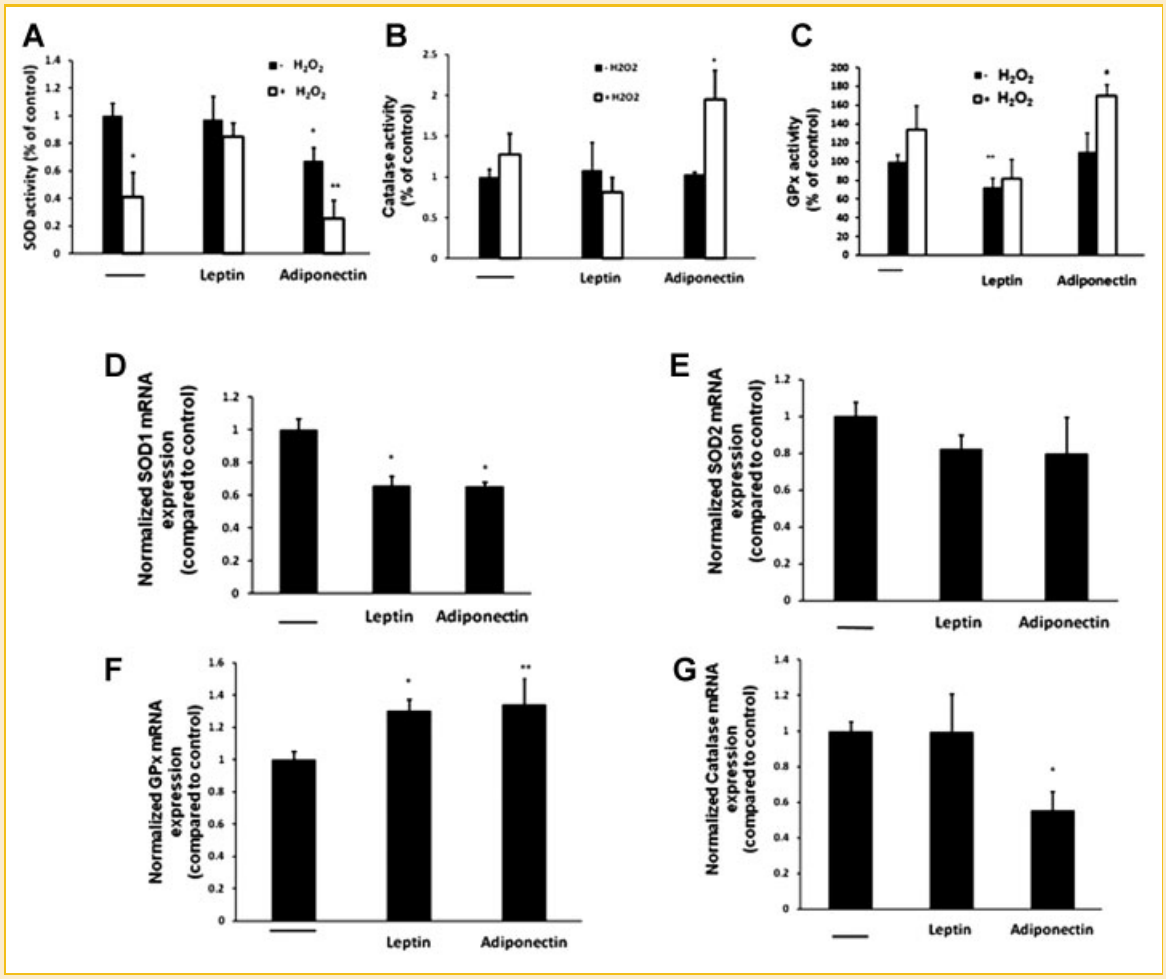

Fig. 3. Leptin and adiponectin regulate activity and mRNA expression of antioxidant enzymes. RINm cells were plated at a density of 10^5 cells/ml and 24 h after plating treated for 40 min with leptin (10 ng/ml,) or adiponectin (10 nM) in the presence or absence of H_2O_2 (70 μM). Activities of SOD (A), catalase (B), and GPx (C) were measured as described in Materials and Methods section. Cells were treated for 5 h with leptin (10 ng/ml) or adiponectin(10 nM). mRNA expression of SOD1 (D) SOD2 (E) GPx (F) and catalase (G), was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT. Results are mean ± SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.005$ compared to untreated cells by Student's *t*-test.

IDENTIFICATION OF SIGNALING MOLECULES MEDIATING ROS PRODUCTION INDUCED BY LEPTIN AND ADIPONECTIN

In order to identify signaling pathways that might mediate ROS generation induced by leptin and adiponectin, we first treated MIN6 cells with specific inhibitors of AMP kinase (AMPK), phosphatidyl inositol 3 kinase (PI3K), or NADPH oxidase (Nox), and followed ROS generation in response to the adipokines. Adiponectin, but not leptin is known to activate AMPK in pancreatic beta-cells [Huypens et al., 2005]. Inhibition of AMPK did not alter leptin- or adiponectin-dependent ROS generation (Fig. 4A). In contrast, leptin, but not adiponectin, is known to activate PI3K in various cell types [Maroni et al., 2005; Perez-Perez et al., 2010; Uddin et al., 2010]. However, inhibition of PI3K not only did not block ROS generation, it actually increased basal and leptin-induced ROS production (Fig. 4B), indicating that PI3K activates signaling pathways that reduce ROS levels. Adiponectin-dependent ROS production was not affected by blockade of PI3K.

DPI is a widely used inhibitor of the Nox family of proteins [Li et al., 2010; Zhuang et al., 2010; Syed et al., 2011]). Inhibition of Nox using DPI blocked both leptin and adiponectin-dependent ROS production (Fig. 4C), indicating that Nox activity is crucial for adipokines-dependent ROS generation.

LEPTIN AND ADIPONECTIN INCREASE PANCREATIC BETA-CELL VIABILITY

The results so far show that leptin and adiponectin increased ROS levels in pancreatic beta-cells by a Nox-dependent mechanism. In order to clarify the physiological relevance of ROS production, we investigated the effects of leptin and adiponectin-induced ROS on beta-cell viability. As shown in Figure 5A, both leptin and adiponectin increased viability of RINm and MIN6 cells. The effects of these adipokines on viability of MIN6 cells was also validated by an additional assay which showed similar results, as is presented in Figure 5B. (CellTiter Cell Proliferation Assay, Promega). This effect

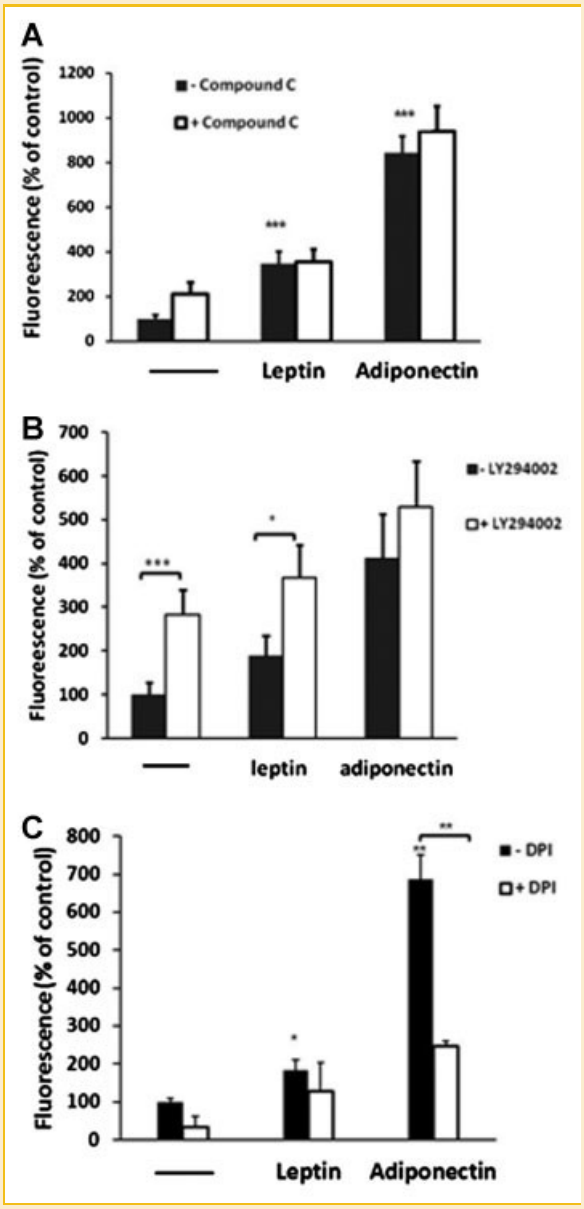

Fig. 4. ROS generation induced by leptin and adiponectin depends on Nox activity. MIN6 cells were plated at a density of 10^5 cells/ml. Twenty four hours after plating cells were treated with leptin (10 ng/ml) or adiponectin (10 nM) in the presence or absence of (A) compound C (5 μM for 30 min), (B) LY294002 (20 μM for 40 min), or (C) diphenylene-iodonium (DPI- 0.5 μM for 40 min). ROS generation was measured as described in Material and Methods section. Results are mean ± SEM of five independent experiments. $^{*}P < 0.05$, $^{***}P < 0.0005$ compared to untreated cells by ANOVA.

was blocked by either NAC (Fig. 5C) or PEG-catalase (Fig. 5D), thus indicating that ROS are required for the molecular pathways leading to increased viability.

LEPTIN AND ADIPONECTIN DO NOT PROTECT BETA-CELLS AGAINST APOPTOTIC STIMULI

Although insulin resistance is the primary defect leading to the development of type 2 diabetes, the clinical symptoms of diabetes result from alteration in beta-cell function and apoptosis leading to reduced beta-cell mass. Several triggers for the induction of apoptosis in beta-cells have been suggested, including lipotoxicity, increased ROS and elevated inflammatory cytokines [Cnop et al., 2005]. The ability of leptin and adiponectin to activate anti-apoptotic pathways in beta-cells is unclear. As shown in Figure 6A, we found that leptin and adiponectin had no protective function against palmitic acid-, cytokine-, or ROS-induced RINm cell death. In addition, in order to clarify whether the increased viability observed following treatment with adipokines (Fig. 5A) was due to increased proliferation rather than to inhibition of apoptosis, we measured caspase-3 activity and DNA fragmentation in cells treated with leptin and adiponectin compared to untreated cells. As shown in Figure 6B,C, neither leptin nor adiponectin reduced apoptosis as determined by either DNA fragmentation or caspase-3 activity. This indicates that the adipokine-induced increase in viability resulted from induction of proliferation.

OXIDATIVE STATE REGULATES INSULIN SECRETION IN BETA-CELLS

In order to study the effects of redox potential on insulin secretion we treated MIN6 cells for 1 h with leptin, adiponectin, NAC, or two concentrations of H_2O_2- 70 and 4 μM, which were found to have opposite effects on beta-cell viability (Fig. 7A). Whereas adiponectin did not affect either basal or glucose stimulated insulin secretion (GSIS), leptin inhibited GSIS. Disturbance of the redox state either to the oxidative (70 μM H_2O_2) or to the reductive state (NAC) inhibited GSIS. NAC reduced basal insulin secretion as well. In contrast, 4 μM H_2O_2 increased GSIS. Preproinsulin mRNA expression was not affected by any of these treatments (Fig. 7B).

DISCUSSION

In our initial experiments, we found that, whereas high concentrations of H_2O_2 induced beta-cell death, low concentrations of this radical increased the viability of these cells. In apparent contradiction to these results, we observed that a high concentration of the antioxidant NAC also inhibited beta-cell growth. These results thus suggest that disturbance in the redox balance either to oxidative or reductive state, rather than H_2O_2 itself, may alter beta-cell viability and growth. Indeed, the effect of low H_2O_2 but not NAC to increase beta-cell viability indicates that a moderate oxidative state may selectively favor proliferation. Among the different ROS, H_2O_2 is the likeliest candidate to act as a second messenger, and has been reported to be involved in physiological signal transduction pathways of several growth factors [Valko et al., 2007; Forman et al., 2010]. Induction of cell proliferation correlates with a transient increase of H_2O_2. H_2O_2 regulates signaling pathways by oxidation of cysteinyl thiol, formation of disulfide bonds, and induction of glutathionylation of cysteine residues in target molecules [Forman et al., 2010].

We further found that both leptin and adiponectin increase intracellular ROS levels in pancreatic beta-cells, as measured both by the oxidation of DCFH-DA to DCF, reflecting total intracellular ROS levels, and by the specific levels of H_2O_2. Whereas leptin and adiponectin each increased total ROS by several folds, only

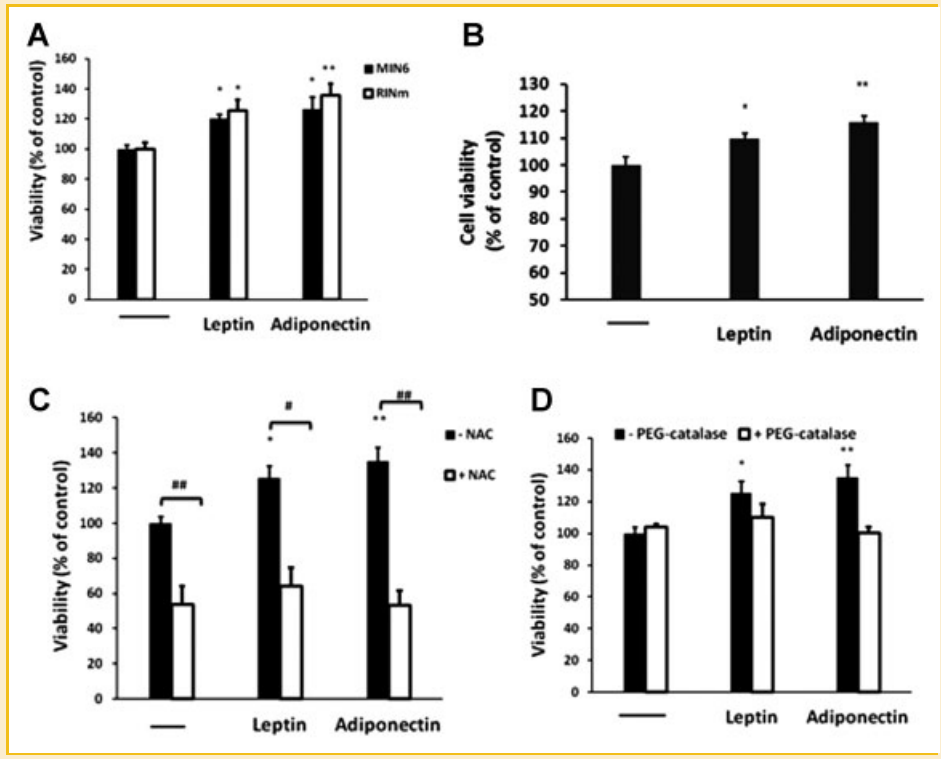

Fig. 5. Leptin and adiponectin increase viability of pancreatic beta-cells. RINm and MIN6 cells were plated at a density of 10^5 cells/ml and (A,B) treated with leptin (10 ng/ml) or adiponectin (10 nM) 24 h after plating. NAC and PEG-catalase abrogate leptin- and adiponectin-induced increase in beta-cell viability. C,D: 24 h after plating RINm cells were treated with leptin (10 ng/ml) or adiponectin (10 nM) in the presence or absence of 10 mM NAC (C) or 50 U/ml PEG-catalase (D). Beta-cell viability was measured 24 h after leptin or adiponectin treatment using the XTT (A, C and D) or CTB (B) reagents as described in Material and Methods section. Results are mean ± SEM of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to untreated cells, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ compared to the appropriate treatment without NAC, by ANOVA.

adiponectin increased H_2O_2 levels, but to a lower magnitude than its effect on total ROS. These results indicate that while H_2O_2 at low concentrations increases proliferation, there are other ROS, such as O_2\cdot−, which are generated by leptin and adiponectin and likely participate in the pro-proliferative effect of leptin and adiponectin as well. Leptin and adiponectin may increase ROS to levels that can regulate signaling pathways, but do not reach the damaging levels associated with oxidative stress, as treatment with physiological concentrations of these adipokines was not accompanied by increased apoptosis or lipid peroxidation products.

Using specific blockers of Nox, AMPK, and PI3K, we found that both leptin- and adiponectin-induced increases in ROS levels were dependent on Nox activity. Nox proteins are membrane-situated enzymes that catalyze the reduction of oxygen to the production of O_2\cdot− using NADPH as an electron donor. Historically, Nox proteins are known to be the source of oxidative burst for phagocyte action. However, it has become clear that Nox enzymes and the subsequently generated ROS, are important contributors to many other signaling pathways in various cell types, including pancreatic beta cells [Newsholme et al., 2009], and are essential for normal cell physiology [Brown and Griendling, 2009]. Thus, the involvement of Nox in adipokine-dependent ROS generation strengthens the assumption that adipokine-induced ROS serve as second messengers for regulation of signaling pathways rather than being byproducts of metabolic pathways. In addition, the inhibitory effect of the Nox inhibitor on ROS production indicates that O_2\cdot−, the product of Nox activity is one of the major ROS produced by leptin and adiponectin. In contrast, AMPK, which is a main signaling molecule mediating adiponectin activity, appears not to be involved in ROS generation. It was reported that leptin action in beta-cells is PI3K, but not AMPK-dependent [Leclerc et al., 2004; Ning et al., 2006]. We found, however, that blockade of PI3K not only did not abrogate leptin-induced increase in ROS, but it even slightly increased it. This indicates that leptin may activate a second antagonistic pathway, mediated by PI3K, which could reduce ROS levels. This pathway could be activated by leptin, but not adiponectin, and may be the reason for the higher increase in ROS levels following adiponectin compared to leptin treatment. This possibility is currently being studied.

The involvement of ROS in beta-cell physiology, in contrast to pathophysiology, has hardly been studied. An indication as to the importance of maintaining ROS in beta-cells may be found in a study showing early onset of type 1 diabetes in mice overexpressing catalase [Li et al., 2006]. Thus, it is possible that the failure of antioxidant supplementations to improve glucose tolerance (as well as other health outcomes) in intervention studies may result from excessive antioxidant intake leading to an unregulated redox state. Regulation of intracellular ROS levels is attributed also to the activity of the cellular antioxidant system which is responsible for the clearance of ROS. We found that adiponectin, which increased

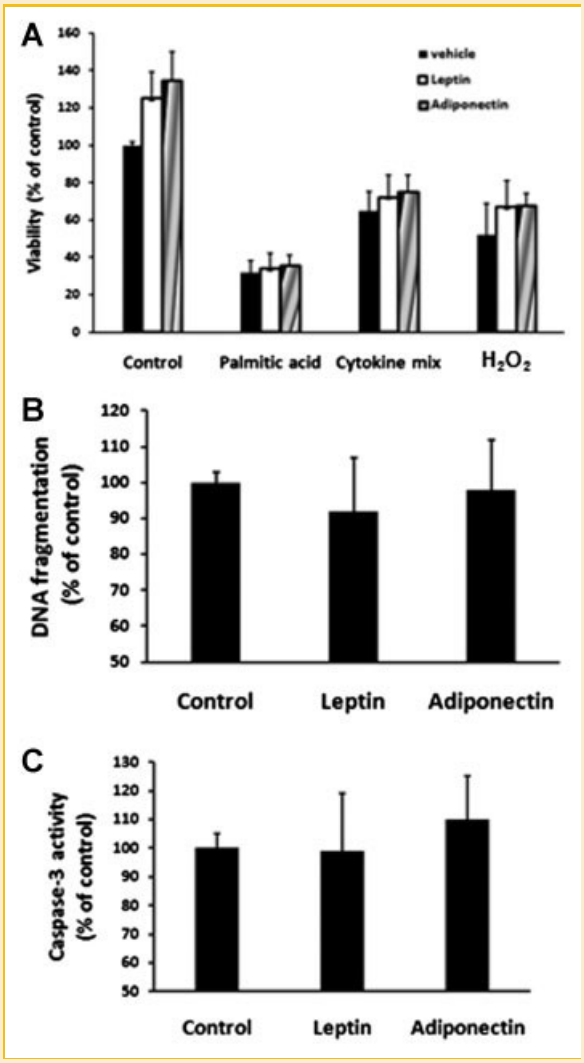

Fig. 6. Apoptosis is not affected by leptin and adiponectin. RINm cells were plated at a density of 3×10^5 cells/ml. A: Cell viability in response to cytokine mix or palmitic acid. Twenty four hours after plating cells were treated with leptin (10 ng/ml) or adiponectin (10 nM) in the presence or absence of palmitic acid (250 μM), cytokine mix or H_2O_2 (70 μM). Beta-cell viability was measured as described in Material and Methods section. B: Leptin and adiponectin do not alter DNA fragmentation. Twenty four hours after plating cells were treated as in (A). DNA fragmentation was measured 24 h later using ELISA cell death detection kit (Roche). C: Leptin and adiponectin do not alter caspase-3 activity. Activity was measured using Caspase-3 colorimetric assay (MBL). Results are mean ± SEM of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to untreated cells by Student's *t*-test.

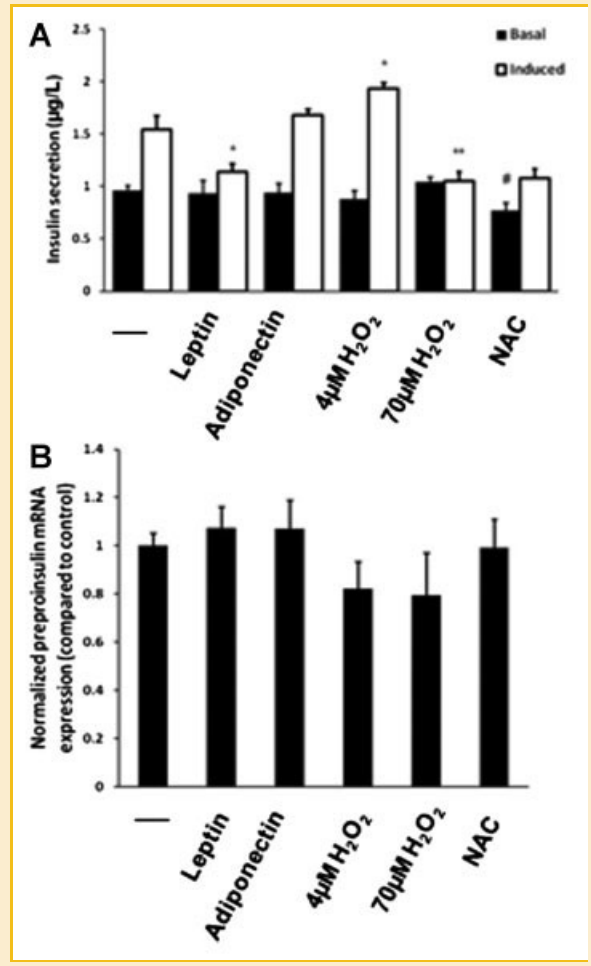

Fig. 7. Oxidative state affects insulin secretion in beta-cells. A: Glucose-Induced Insulin Secretion. MIN6 pancreatic beta-cells were grown as described and treated for 1 h with leptin (10 ng/ml), adiponectin (10 nM), H_2O_2 at low (4 μM) or high (70 μM) dose, or NAC (10 mM). Supernatant was collected at the basal state from nonstimulated cells and after glucose induction of insulin secretion. The induction of insulin secretion and measurement of insulin secretion were performed as described in the Materials and Methods section. Results are mean ± SEM of three independent experiments ($^{\#}P < 0.05$ compared to basal insulin secretion in untreated cells, $^{*}P < 0.05$, $^{**}P < 0.005$ compared to glucose-induced insulin secretion in untreated cells. B: Preproinsulin mRNA expression. The change in expression was measured by real-time PCR following 5 h of treatment with 10 ng/ml leptin, 10 nM adiponectin, H_2O_2 at low (4 μM) or high (70 μM) dose, or 10 mM NAC. Results were normalized to the expression of HPRT (housekeeping gene). Results are mean ± SEM of three independent experiments.

ROS level, decreased SOD activity and increased catalase and GPx activities in the presence of damaging levels of H_2O_2. Thus, we suggest that adiponectin couples increased ROS generation to activation of the antioxidant enzyme system in order to prevent accumulation of cellular ROS, which may lead to cytotoxic effects. This idea is supported by our results showing that the increased ROS levels induced by adiponectin were not followed by increased apoptosis. In addition, it was suggested [Savini et al., 2003] that adiponectin activates antioxidant enzymes in a way that increases the tolerance of cells to oxidative stress. Our results, however, do not support these findings, as we found that neither adiponectin nor leptin protected beta-cells against oxidative stress, even when given 24 h before its induction (data not shown). Thus, the physiological relevance of leptin and adiponectin-dependent regulation of antioxidant enzymes needs to be better clarified.

While the anti-atherogenic and anti-inflammatory properties of adiponectin are well documented, the physiological effects of adiponectin on pancreatic beta-cell function are poorly understood. Adiponectin has been reported to augment [Gu et al., 2006], decrease [Winzell et al., 2004] or, as supported by our results, not to be

involved [Staiger et al., 2005a] in insulin secretion. In addition, the ability of adiponectin to protect beta-cells against apoptotic stimuli is also controversial [Staiger et al., 2005; Lin et al., 2009]. We confirmed that apoptosis induced by palmitic-acid, H_2O_2 and cytokines is not abrogated by adiponectin. On the other hand, we showed in two different cell-lines that adiponectin increased pancreatic beta-cell viability. These results are in accord with two recently published studies reporting increased viability of MIN6 and mouse islets [Brown et al., 2010; Wijesekara et al., 2010] following adiponectin treatment.

We also showed that leptin has effects similar to adiponectin on beta-cell viability, as has been shown in other studies [Tanabe et al., 1997; Okuya et al., 2001]. The findings that caspase-3 activity and DNA fragmentation were not inhibited by leptin and adiponectin are consistent with those showing that leptin and adiponectin increased viability via induction of proliferation rather than inhibition of apoptotic pathways. We found that the induction of proliferation by leptin and adiponectin depends on the existence of low levels of ROS, as treatment with NAC and PEG-catalase abrogated the effect of leptin and adiponectin on beta-cell viability. The possibility that antioxidant supplementation may affect beta-cell mass and interfere with leptin and adiponectin function in-vivo remains to be clarified.

Insulin secretion is affected as well by alteration of the redox potential in cells [Robertson, 2006]. In this regard, we found that a short exposure to either H_2O_2 or NAC, at a concentration that alters cell viability, inhibited insulin secretion. Oxidative stress is known to interrupt insulin secretion secondary to oxidative cell damage as well as by activation of several stress-sensitive signaling pathways [Hou et al., 2008]. Similarly, NAC, a known scavenger of ROS, inhibited basal insulin secretion and completely blocked GSIS. Thus, by completely eliminating cellular ROS, insulin secretion is depressed. In accord with that, we found that a low concentration of H_2O_2 slightly increased insulin secretion in RINm cells, as was also shown in INS-1 cells [Pi et al., 2007] indicating that 'non-damaging' levels of ROS may be required for the propagation of the pathways leading to insulin secretion. Whereas the effect of leptin and adiponectin on beta-cell viability is ROS-dependent, we assume that leptin regulation of insulin secretion occurs by a ROS-independent mechanism. Thus, we found that while low concentration of H_2O_2 increases insulin secretion, the ROS-independent mechanism activated by leptin inhibits the ROS-dependent induction of GSIS, presumably due to moderate ROS production by leptin. Even though adiponectin increases ROS generation, this adipokine has no effect on insulin secretion, as shown in several other studies. The difference between the effect of H_2O_2 and the adipokines on insulin secretion may also indicate that leptin and adiponectin increase ROS other than H_2O_2.

In summary, the results of this study show that leptin and adiponectin have positive effects on beta-cell viability. Moreover, the findings demonstrate the importance of maintaining well-regulated redox balance for the induction of beta-cell proliferation.

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