

Adiponectin prevents islet ischemia-reperfusion injury through the COX2-TNF α -NF- κ B-dependent signal transduction pathway in mice

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Abstract

Islets are exceptionally susceptible to ischemia–reperfusion injury, an increased incidence of primary graft nonfunctionality, and β -cell death during a transplant procedure. Therefore, islets require protection during the early stages of the transplant procedure. Based on the beneficial vascular and anti-inflammatory activity of adiponectin, we hypothesize that adiponectin protects islet cells against ischemia–reperfusion injury and graft dysfunction after transplantation. To examine the effects of adiponectin on the resistance of islet ischemia–reperfusion injury, we used the islet hypoxia–reoxygenation injury model and performed kidney subcapsular syngeneic islet transplants to assess the islets' vitality and function. Furthermore, we utilized lipopolysaccharide (LPS)-induced or tumor necrosis factor α (TNF α)-induced damage to islet cells to model the inflammation of post-transplant ischemia–reperfusion injury and transplanted islets in adiponectin knockout mice to explore whether the protective action of adiponectin is involved in TNF α production and nuclear transcription factor- κ B (NF- κ B) activation. Adiponectin suppressed TNF α production and I κ B- α phosphorylation; decreased hypoxia–reoxygenation and LPS-induced and TNF α -induced islet apoptosis; and improved islet function *in vivo* and *in vitro*. Our results demonstrate that adiponectin protects the islet from injury. We show that islet protection occurs in response to ischemia–reperfusion and is dependent on the suppression of islet production by TNF α through cyclooxygenase 2 and the inhibition of the TNF α -induced NF- κ B activation pathways.

Key Words

- ▶ ischemia–reperfusion
- ▶ islet transplantation
- ▶ adiponectin

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Introduction

Islet transplantation for the treatment of type 1 diabetes mellitus is limited in its clinical application mainly due to early loss of the transplanted islets, resulting in low

transplantation efficiency (Matsuoka *et al.* 2010). The islets are exceptionally susceptible to ischemia–reperfusion injury (Sklavos *et al.* 2010). Hypoxia–reoxygenation is the

leading cause of β -cell death during islet preparation, implantation, and revascularization, with the highest percentage of islet graft loss and dysfunction occurring just days after transplantation (Montaña *et al.* 1993, Davalli *et al.* 1996, Sklavos *et al.* 2010). The reduction and the repair of the ischemia–reperfusion injury at its early stage of transplantation has become the key link that could prolong graft function and survival and improve the success rate of clinical islet transplantation.

Adiponectin is the most abundant adipose-specific adipokine that acts as an anti-obesity and anti-inflammatory hormone (Yang *et al.* 2001, Elias-Miro *et al.* 2011). A number of reports suggest that the beneficial vascular and anti-inflammatory activity of adiponectin might contribute to its therapeutic potential during ischemia–reperfusion injury (Goldstein *et al.* 2009, Ohashi *et al.* 2012). Recently, several studies have demonstrated that exogenous adiponectin protects against myocardial (Kondo *et al.* 2010), cerebral (Chen *et al.* 2009), vascular (Goldstein *et al.* 2009), hepatic (Massip-Salcedo *et al.* 2008), and renal (Cheng *et al.* 2012) apoptosis/necrosis during ischemia and reperfusion *in vivo* and *in vitro*. The mechanism behind these effects may be related to AMP-activated protein kinase, tumor necrosis factor α (TNF α), cyclooxygenase 2 (COX2), and transcription nuclear factor- κ B (NF- κ B), as well as inhibition of the inducible isoform of NO synthase.

Ischemia–reperfusion injury is the key factor associated with islet graft dysfunction at early stages of the transplant procedure. Islet transplantation may benefit from the agents that protect ischemia–reperfusion injuries and inhibit the early inflammatory cascades for the preservation of islet function. Based on these important biological characteristics of adiponectin, we hypothesize that the inhibition of ischemia–reperfusion injury progression via adiponectin activity is a potential treatment in islet transplantation procedures to decrease the incidence of islet loss in functional grafts and islet viability after transplantation. To examine the effects of adiponectin on the resistance to islet ischemia–reperfusion injury *in vivo* and *in vitro*, we induced islet apoptosis and injury by hypoxia–reoxygenation in the presence or absence of adiponectin, which was incubated *in vitro*, to assess the vitality and insulin secretion capability of islets. Additionally, we performed kidney subcapsular, suboptimal (100 islets/recipient), and marginal mass (200 islets/recipient) syngeneic islet transplants in diabetic recipients, both in the presence or in the absence of adiponectin, to assess transplantation efficiency and islet function. To further investigate the pathway and molecular mechanisms

underlying the effect of adiponectin on islet cells, we utilized the lipopolysaccharide (LPS)-induced or TNF α -induced islet damage model to mimic the inflammation of post-transplanted ischemia–reperfusion injury. Both *in vitro* and in transplanted islets in adiponectin knockout (APN-KO) mice, we tested for the presence or the absence of adiponectin to explore whether the protective action of adiponectin is involved in TNF α production and NF- κ B activation. Our results demonstrate that adiponectin activity protects the islet from injury in response to ischemia–reperfusion through the suppression of the islet production of TNF α and inhibition of TNF α -induced NF- κ B activation.

Materials and methods

Islet isolation and culture

Male C57BL/6 islets were isolated using established methods (Luo *et al.* 2010). Briefly, the pancreas was distended with 2.5 ml, 0.5 mg/ml collagenase XI (Sigma) solution and digested for 17 min at 37 °C in a water bath. The islets were purified by density gradient centrifugation. The islets were collected and washed with Hank's solution containing 1% BSA (Roche), then stained with dithizone (Sigma), and counted under a microscope. The islets were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin.

In vitro hypoxia–reoxygenation injury model

Islets were cultured under hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂) for 8 h in a six-well plate with or without 10 μ g/ml adiponectin. Next, they were transferred to ordinary conditions and cultured for a further 16 h. The plates were analyzed immediately. Islets were incubated with the anti-adiponectin MAB ANOC 9104 or the nonimmune mouse IgG (30 mg/ml) with a biotinylated adiponectin treatment. The islets that were exposed to hypoxia were compared with the control islets that were cultured for the same duration under ordinary conditions.

In vitro inflammation model induced by LPS or TNF α

To mimic the inflammation of islets post-transplant, we utilized the LPS- or TNF α -induced islet damage test. We pretreated islet cultures for 18 h in the presence or absence of adiponectin (10 μ g/ml) and subjected them to LPS (1 mg/ml) stimulation for 6 h or subjected them to

recombinant mouse TNF α (10 U/ml; R&D Systems) stimulation for 48 h. In other experiments, we preincubated the islets with the COX2 inhibitor NS398 (2 mM), the I κ -B α phosphorylation inhibitor Bay 11-7085 (10 μ M), or vehicle for 1 h before adiponectin treatment.

Determination of islet cell apoptosis and viability

The cell apoptosis rate was determined by flow cytometry (FCM) assay using an apoptosis detection kit (Keygen, Nanjing, China). Islet cells were dissociated into single cells by incubation with trypsin–EDTA for 7 min. The dissociated islet cells were incubated with propidium iodide (PI) and annexin V-FITC (annexin V) for 15 min at room temperature. Next, the samples were analyzed via FCM (Beckman Elite EXP). Dual-parameter FCM was used to determine the number of apoptotic cells (annexin V/PI double-positive cells). In addition, the activity of caspase-3 in islets was examined by western blot analysis. Islet viability was determined using fluorescein diacetate (FDA; Sigma) and PI (Sigma). FDA is metabolized by living cells and fluoresces green when excited, and PI penetrates into necrotic cells and labels nuclei, which fluoresce red when excited. The empirical ratio between the number of green cells and the PI positively stained nuclei of cells indicates the percentage viability.

Glucose-stimulated insulin secretion and insulin content measurement

Handpicked islets were subjected to a static glucose challenge in Krebs–Ringer bicarbonate buffer (KRBB; pH 7.35) containing 10 mmol/l HEPES and 0.5% BSA (Sigma). After conditioning, the islets were incubated in KRBB containing low (2.8 mmol/l) and high (20 mmol/l) glucose concentrations for 1 h. At the end of the glucose challenge, the insulin levels were measured by ELISA (Linco Research, St Charles, MI, USA). The stimulation index (SI) for each experimental condition was calculated as the ratio of insulin released in high vs low glucose concentrations.

Measurement of TNF α

We quantified the concentration of TNF α in the culture medium with the use of ELISA Kits (R&D Systems). The number of TNF α -producing cells was determined using phycoerythrin-labeled anti-TNF α (BD Pharmingen, San Diego, CA, USA) as described previously (Jung *et al.* 1993), with modifications. Briefly, islet cells were incubated in culture media containing 10 μ g/ml brefeldin A (a Golgi

inhibitor) for 4 h at 37 °C/5% CO₂. Following two washes, cells were incubated with fluorescent-labeled anti-TNF α for 30 min at room temperature. After washing, cells were fixed in 0.3 ml, 2% formaldehyde and kept at 4 °C in the dark until acquisition. Analyses were performed using a flow cytometer (Becton Dickinson, Mountain View, CA, USA).

NF- κ B (p65) DNA-binding activity assay

NF- κ B (p65) DNA-binding activity in islets was examined using the TransAMTM ELISA Kit (Active Motif, Carlsbad, CA, USA) as described by Seki *et al.* (2010). In brief, 0.5 μ g nuclear extract was subjected to the binding of NF- κ B to an immobilized consensus sequence (5'-GG-GACTTTC-3') in a 96-well plate to which the primary and secondary antibodies were added. After a colorimetric reaction, the samples were measured in a spectrophotometer at a wavelength of 450 nm. Recombinant NF- κ B p65 (Active Motif) was used as a protein standard. The DNA-binding specificity was assessed using wild-type or mutated oligonucleotides.

Western blot analysis

Whole-cell lysates were resolved on 10% SDS–PAGE gels followed by electrophoretic transfer to nitrocellulose membranes. After transfer to membranes, we performed an immunoblot analysis with the indicated primary antibodies at a 1:1000 dilution. This was followed by incubation with the secondary antibody conjugated with HRP at a 1:5000 dilution. We used the ECL-PLUS Western Blotting Detection Kit (Amersham Pharmacia Biotech) for detection. Primary antibodies were an anti-TNF α rabbit polyclonal antibody, anti-COX2 rabbit polyclonal antibody, anti-prostaglandin E₂ (PGE₂) rabbit polyclonal antibody, anti-phospho I κ B- α rabbit polyclonal antibody, anti-phospho JNK rabbit polyclonal antibody, anti-phospho Akt kinase rabbit polyclonal antibody, anti-caspase-3 MAB (all antibodies supplied by Abcam), and anti-GAPDH MAB (Biogenesis, Stinford Flood, UK).

Syngeneic islet transplantation

Eight- to 12-week-old C57BL/6 male mice were used as donors. APN-KO and wild-type C57BL/6 mice (7–8 weeks old) were used as recipients. APN-KO mice were generated as described previously (Maeda *et al.* 2002) and maintained in a C57BL/6J background. Syngeneic recipients were rendered diabetic by i.v. administration of 200 mg/kg streptozotocin

(Sigma). The animals were declared diabetic after two consecutive blood glucose measurements >250 mg/dl. Using a dissecting microscope, islets were simultaneously picked, counted, and redistributed onto Petri dishes containing 100 or 200 islets of 150–250 μ m in diameter each. Each recipient was transplanted with suboptimal (100 islets/recipient) or marginal mass (200 islets/recipient) syngeneic islets. These syngeneic islets were inserted under the kidney capsule as described in Sklavos *et al.* (2010). Transplant recipients were given a single adiponectin or vehicle injection (1 mg/kg body weight i.v.) on the day of transplant. Recipients of suboptimal mass syngeneic islets had their blood glucose monitored approximately every other day for 28 days. Recipients of marginal mass syngeneic islets were subjected to a fasting intraperitoneal glucose tolerance test (IPGTT) on postoperative day 7. After 18 h of fasting, the mice were injected with a 10 μ l/g body weight dose of sterile-filtered 20% glucose solution in distilled H₂O. Blood glucose readings were taken at 0, 30, 60, 90, and 120 min after glucose injection. Nephrectomies were performed on postoperative day 8 to confirm that the islet graft had been functional, which was confirmed by the return to blood glucose levels >250 mg/dl on 2 consecutive days.

Statistical analysis

All data are expressed as mean \pm S.E.M. The differences between mean values were determined by the Student's *t*-test for singular comparisons and by the one-way ANOVA for multiple comparisons. The area under the curve (AUC) was determined using the trapezoidal rule. Kaplan–Meier survival plots were analyzed by the log-rank test. All statistical analyses were performed with SPSS using $P < 0.05$ to achieve significance.

Results

Adiponectin decreases apoptosis in islets and improves islet function in an *in vitro* hypoxia–reoxygenation injury model

After the islet cells were cultured with or without adiponectin in hypoxia–reoxygenation for 24 h, islet apoptosis was determined by a FCM assay, the activity of caspase-3 was examined by western blot analysis, the islet viability was determined by PI/FDA staining, and the islet function was determined by glucose-stimulated insulin secretion (GSIS). The results of two independent experiments showed that the apoptotic rate of islet cells (annexin V/PI double-positive cells) was lower when

they were cultured with adiponectin than when they were cultured alone (12.7 ± 1.4 vs $27.5 \pm 2.1\%$, $P < 0.01$) (Fig. 1A and B). The activity of caspase-3 increased significantly in hypoxia–reoxygenation-induced apoptosis in islets ($P < 0.01$; Fig. 2A and B). However, adiponectin could protect islets from apoptosis ($P < 0.01$; Figs 1A and B, 2A and B). Double-fluorescence viability was performed to determine whether DNA content might be affected by the presence of dead non-degraded cells in lower proportion within the adiponectin-treated cells. The results indicate that the control showed higher dead cell content after hypoxia–reoxygenation and that the percentage of viability was higher in the adiponectin-treated group (Supplementary Figure 1a, see section on supplementary data given at the end of this article). The function of GSIS on islet cells decreased after exposure to hypoxia–reoxygenation. Instead, the average glucose SI of the islets treated with adiponectin was significantly higher than that of the islets alone (GSIS: 2.32 ± 0.17 vs 1.47 ± 0.11 , $P < 0.01$; Fig. 1C). Additionally, the effect of adiponectin on islet hypoxia–reoxygenation injury was inhibited by an anti-adiponectin antibody (Figs 1A, B and C, 2A and B).

Adiponectin improves islet function and survival *in vivo*

We investigated whether adiponectin could prevent the early loss of transplanted islets. Blood glucose monitoring showed that adiponectin significantly enhanced islet survival post-transplant in the suboptimal mass islet transplantation (Fig. 1B and E). The ability to reach euglycemia (<200 mg/dl) within 14 days in suboptimal mass islet transplantation was as follows: 12 of 20 mice (60%) for the adiponectin group and 5 of 20 mice (25%) for the control group (Fig. 1F). Next, we transplanted marginal mass islets into diabetic mice to compare their functional performance post-transplant using IPGTT. The results show that there was little difference in IPGTT profiles between the adiponectin group and the normal group at 7 days post-transplant (Fig. 1G). By contrast, control animals exhibited an increase in peak blood glucose values during IPGTT. When the AUC was calculated, the adiponectin-treated group's animals had a significantly lower AUC compared with control animals. These results suggest that adiponectin improves islet function or survival *in vivo*.

Reduced production of TNF α by adiponectin

Because TNF α plays a key role in mediating ischemia–reperfusion injury and acts as a marker cytokine during

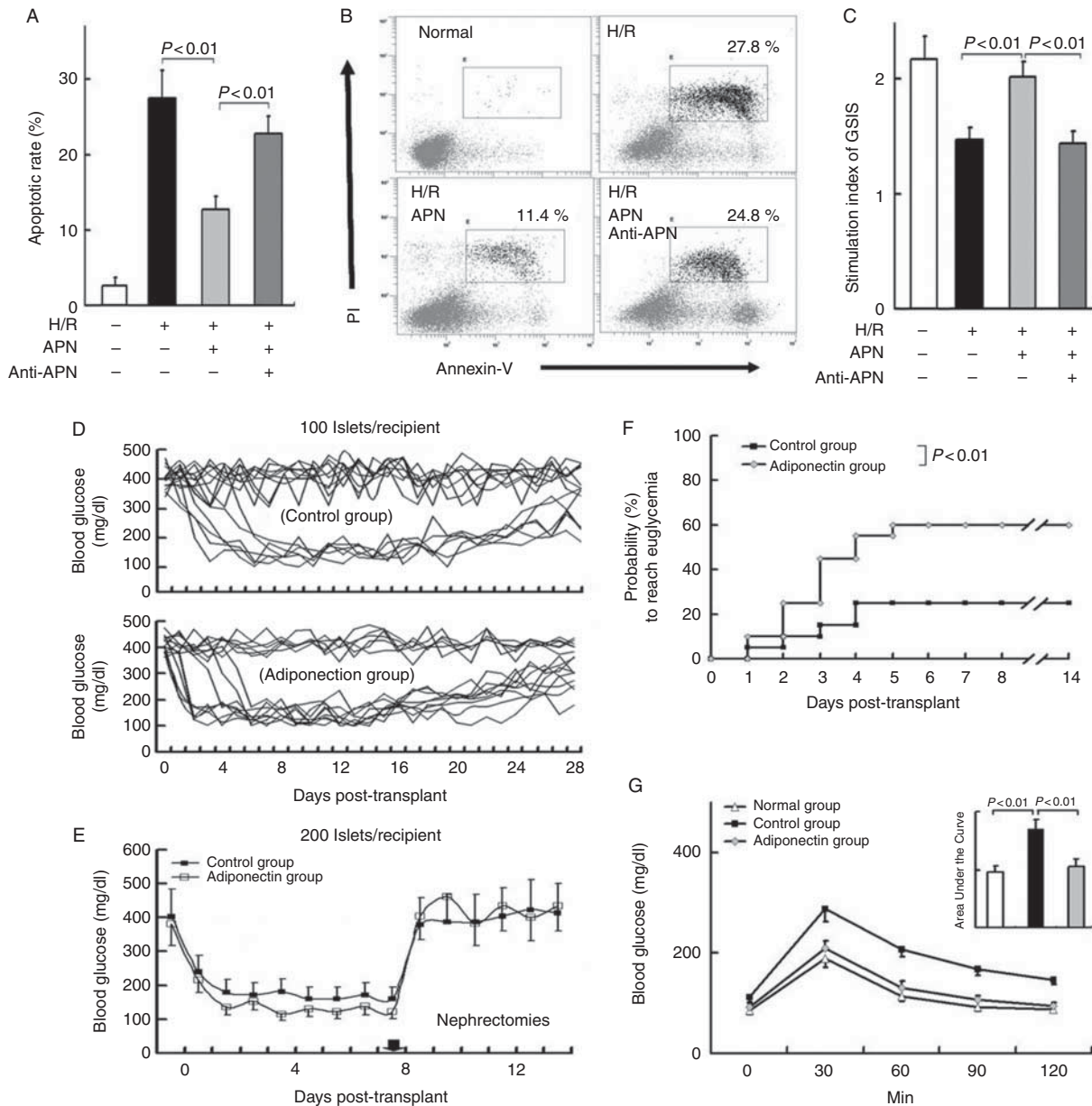


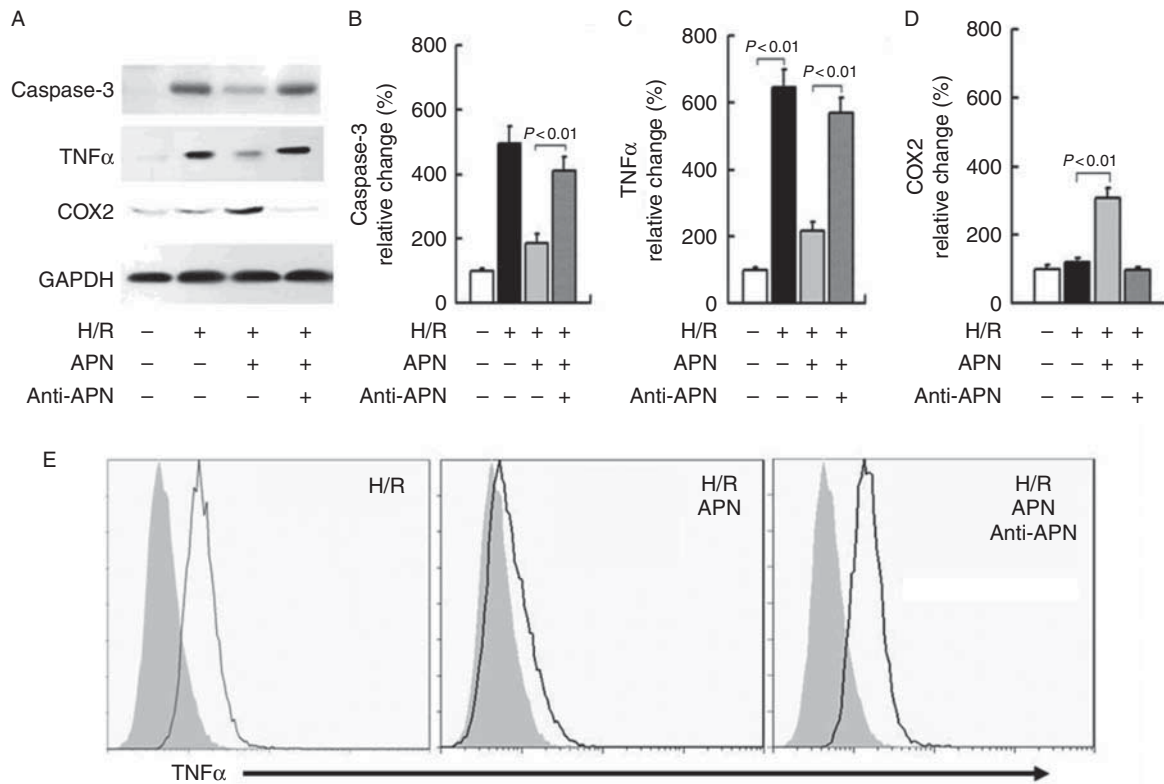
Figure 1

Adiponectin leads to decreased islet apoptosis and improves islet function and survival in an *in vitro* hypoxia-reoxygenation injury model and *in vivo* syngeneic islet transplantation model. The apoptotic rate (A) of islet cells (annexin V/PI double-positive cells), representative pictures (B) of islet apoptosis by FCM assay, and the function of GSIS (C) of islets. The islets were treated with 10 μ g/ml adiponectin (anti-adiponectin monoclonal antibody ANOC 9104 or nonimmune mouse IgG (30 mg/ml) with a biotinylated adiponectin treatment) or vehicle in serum-free media for 24 h under normoxic conditions or 24 h under hypoxic -reoxygenation conditions ($n=6$). Suboptimal and marginal mass islet grafts were transplanted under

organ rejection (Shibata *et al.* 2005, Siasos *et al.* 2012), we assessed insular levels of TNF α *in vitro* and *in vivo*. Immunohistochemical studies revealed that production of TNF α in islet grafts at 7 days post-transplant in

the kidney capsule in syngeneic diabetic recipients. Blood glucose monitoring (D: 100 islets/recipient; E: 200 islets/recipient) showed that adiponectin significantly enhanced islet survival post-transplant. The probability (F) to reach euglycemia (<200 mg/dl) within 7 days in suboptimal syngeneic islets for transplantation (100 islets/recipient) ($n=20$), and IPGTT profiles (G) at 7 days post-transplant in marginal mass (200 islets/recipient) syngeneic islets (blood glucose readings were taken at 0, 30, 60, 90, and 120 min after glucose injection) ($n=5$). Transplant recipients were given a single adiponectin or vehicle injection (1 mg/kg body weight i.v.) on the day of transplant.

adiponectin-deficient mice was increased compared with the mice treated by adiponectin (Supplementary Figure 1b). We then confirmed these results using examination of the serum TNF α level. The results also

**Figure 2**

Adiponectin reduces the production of TNF- α in islets induced by hypoxia-reoxygenation. The islets were treated with 10 μ g/ml adiponectin (anti-adiponectin monoclonal antibody ANOC 9104 or nonimmune mouse IgG (30 mg/ml) with a biotinylated adiponectin treatment) or vehicle in serum-free media for 24 h under normoxic conditions or 24 h under

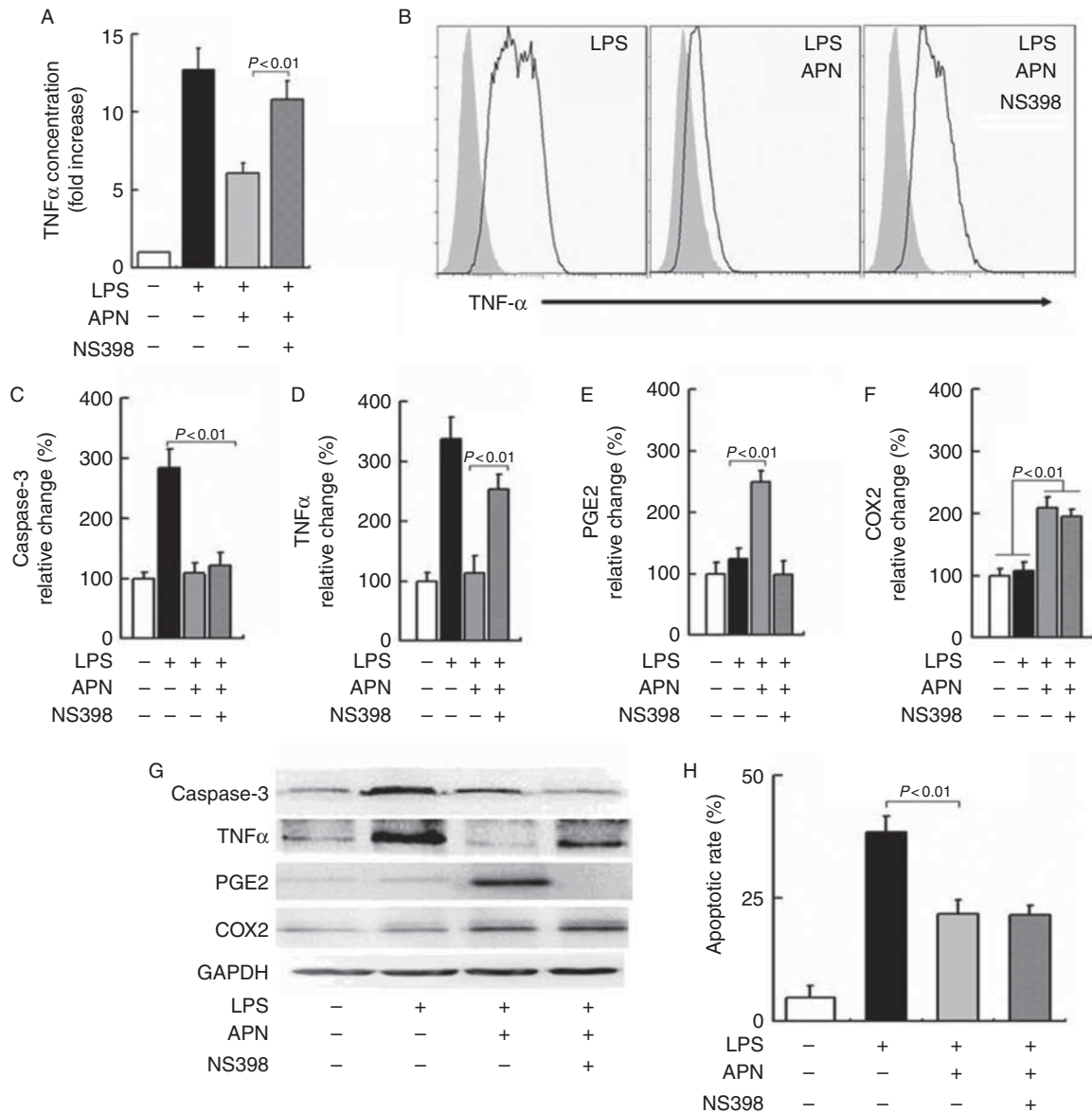
hypoxic-reoxygenation conditions ($n=6$). The relative change effect of adiponectin on the expression of Caspase-3 (B), TNF- α (C) and COX-2 (D) were quantified and expressed relative to untreated group, under reoxygenation-induced conditions in islets (A); (E) The number of TNF- α -producing cells was determined by the FCM assay.

show that the adiponectin can significantly reduce the level of TNF α (Supplementary Figure 1c). Western blot (Fig. 2A, B and C) and FCM analyses (Fig. 2E) show that hypoxia-reoxygenation leads to an increase in the levels of TNF α in islets. Treatment with 10 μ g/ml adiponectin revealed a marked reduction in TNF α , but the suppressive effect of adiponectin was inhibited by an anti-adiponectin MAB. Moreover, our results showed that adiponectin has a dose-dependent response on TNF α generation (Supplementary Figure 2, see section on supplementary data given at the end of this article). We also measured mRNA levels of *IL1 β* and *IL6* in islets. Both cytokines were upregulated after hypoxia and reoxygenation but did not differ between adiponectin-treated and control groups.

Adiponectin inhibits TNF α production through COX2

To test the effects of adiponectin on TNF α production, islets were stimulated by LPS *in vitro* for 6 h in the presence

or absence of adiponectin. The concentration of TNF α in the culture medium was determined by ELISA. LPS exposure increased the secretion of TNF α by 12.7-fold in islets, and treatment with adiponectin markedly inhibited LPS-induced production of TNF α in islets (Fig. 3A). PGE₂ inhibits LPS-induced production of TNF α (Matsuda *et al.* 2005, Parazzoli *et al.* 2012). We found that adiponectin inhibited LPS-induced production of TNF α (Fig. 3A, B, D and G) and enhanced production of PGE₂ in LPS-stimulated islets (Fig. 3E and G). We also found that adiponectin increased the expression of COX2, which is the rate-limiting step for PGE₂ synthesis, in islets stimulated by LPS or hypoxia-reoxygenation (Fig. 2A and D). Therefore, we assessed whether the synthesis of PGE₂ regulated by adiponectin is dependent on COX2 in islets. The adiponectin-stimulated production of PGE₂ was inhibited by the selective COX2 inhibitor NS398 (Fig. 3E and G). Additionally, the COX2 inhibitor NS398 blocked the suppressive effect of adiponectin on the LPS-induced

**Figure 3**

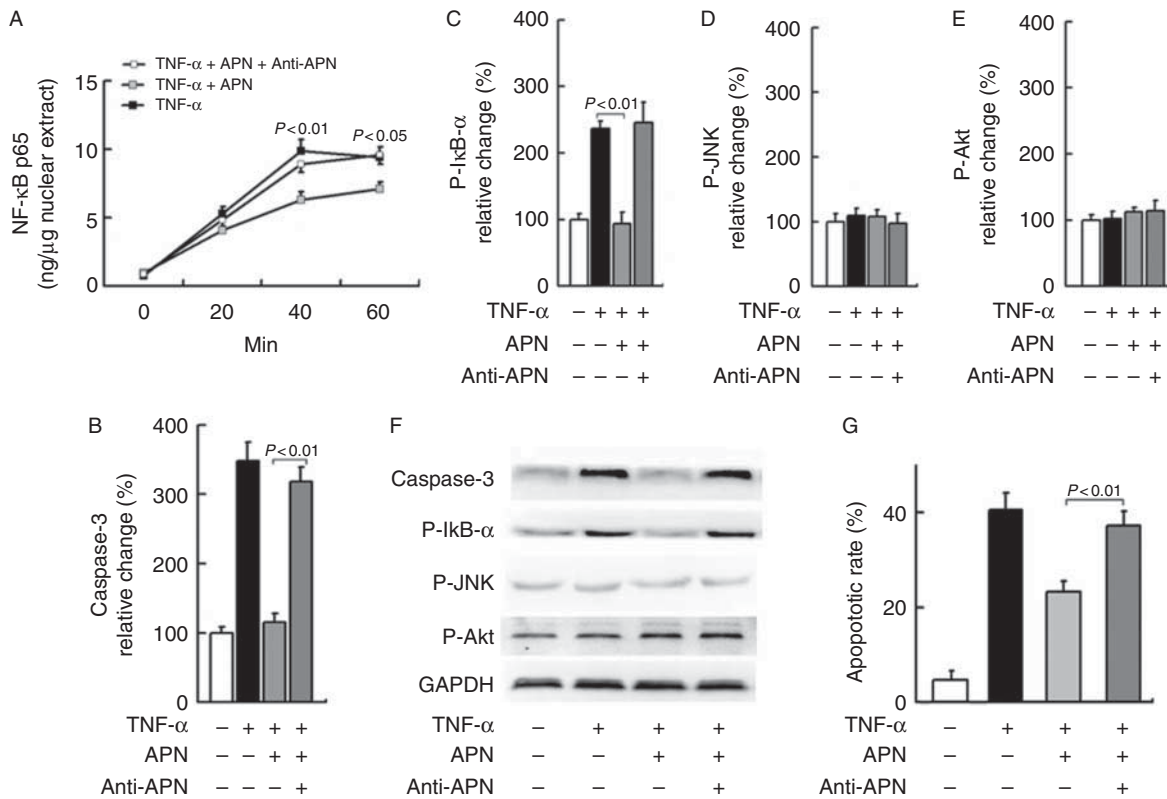
Adiponectin suppresses the LPS-induced secretion of TNF- α from islets through a COX-2-dependent pathway. Islets were cultured for 18 h in the presence or absence of adiponectin (10 μ g/ml) and subjected to LPS (1 mg/ml) stimulation for 6 h. Pretreatment with the COX-2 inhibitor NS398 blocked adiponectin-stimulated production of PGE₂ in the presence or absence of LPS. ($n=4-6$) (A) TNF- α concentrations in media were

determined by ELISA; (B) The number of TNF- α -producing cells was determined by the FCM assay. The relative change effect of adiponectin on the expression of Caspase-3 (C), TNF- α (D), COX-2 (E) and PGE₂ (F) in LPS-induced islets (G) were quantified and expressed relative to untreated group. The apoptotic rate (H) of islet cells (annexin V/PI double-positive cells) by the FCM assay.

production of TNF α (Fig. 3A, B, D and G). Collectively, these data suggest that adiponectin suppresses the secretion of TNF α through a COX2-PGE₂ pathway. However, these were unlike our previous data. Abrogation of the adiponectin activity by the inhibition of COX2 was not associated with an increase in the activity of caspase-3 and apoptosis in islets induced by LPS (Fig. 3C, G and H).

Adiponectin specifically inhibits the NF- κ B pathway that is stimulated by TNF α

NF- κ B plays an important role in the transcriptional regulation stimulated by TNF α (Malinin *et al.* 1997, Tak & Firestein 2001). We examined the effect of adiponectin on TNF α -induced NF- κ B activation in islets to determine the levels of NF- κ B (p65), DNA-binding activity, and the

**Figure 4**

Effects of adiponectin on inhibition of the NF- κ B pathway stimulated by TNF- α . Islets were cultured for 18 h in the presence or absence of 10 μ g/ml adiponectin (anti-adiponectin monoclonal antibody or nonimmune mouse IgG (30 mg/ml) with a biotinylated adiponectin treatment) and were then subjected to recombinant mouse TNF- α (10 U/ml R&D systems) stimulation for 48 h ($n=4-5$). (A) Inhibitory effect of adiponectin on NF- κ B DNA-binding activity in islets at 0, 20, 40, 60 min after TNF- α addition; nuclear

extracts of islets homogenates were analyzed using ELISA; (E) The active form of caspase-3 and phosphorylated form of I κ B- α , JNK, or Akt kinase induced by TNF- α (10 U/ml) with or without adiponectin at 40 minutes after TNF- α stimulation (F) and caspase-3 (B) and I κ B- α (C), JNK (D) and Akt (E) phosphorylation relative changes were quantified and expressed relative to untreated group. The apoptotic rate (G) of islet cells (annexin V/PI double-positive cells) by the FCM assay.

phosphorylation of I κ B- α . Adiponectin treatment decreased the amount of the NF- κ B (p65) DNA-binding complex induced by TNF α stimulation (Fig. 4A). Without adiponectin treatment, the TNF α -induced NF- κ B (p65) DNA-binding activity peaked at 40 min after TNF α stimulation. Furthermore, we examined the phosphorylation of I κ B- α in islets at 40 min after TNF α stimulation. Our results show that adiponectin treatment significantly suppressed TNF α -stimulated I κ B- α phosphorylation (Fig. 4C and F). Next, we determined whether adiponectin affected the JNK and Akt kinase pathways stimulated by TNF α (Fig. 4B, E and F). In contrast to I κ B- α , adiponectin treatment had no effect on TNF α -mediated phosphorylation of these kinases. In addition, the suppressive effect of adiponectin on TNF α -induced NF- κ B activation was blocked by the anti-adiponectin MAB (Fig. 4A and C) and was associated with increased activity of caspase-3 (Fig. 4B and F) and apoptosis in cultured islets induced by

TNF α (Fig. 4G). These results indicate that adiponectin specifically suppresses TNF α -induced NF- κ B activation.

Discussion

Our data provide evidence that adiponectin confers resistance to islet ischemia-reperfusion injury. When islets were incubated with adiponectin, it maintained the vitality and insulin secretion capability of the islet cells and decreased the rate of apoptosis and injury induced by hypoxia-reoxygenation *in vitro*. The adiponectin treatment showed improvement in islet function after the post-transplant engraftment as measured by IPGTTs. Additionally, our study suggests that adiponectin increased 2.25-fold with the ability to reach euglycemia within 7 days in suboptimal islet transplantation. However, the pathway and molecular mechanisms underlying the effect of adiponectin on islet cells needs further

research. Our study demonstrates that adiponectin protects the islet from injury in response to ischemia–reperfusion through at least two mechanisms: suppression of islet production of TNF α through COX2 and specifically inhibited TNF α -induced NF- κ B activation.

Here, we show that adiponectin functions to suppress islet production of TNF α *in vitro* and *in vivo*. The increased production of proinflammatory cytokines is an important component of ischemia/reperfusion injury, and TNF α was identified as a marker cytokine during islet transplant rejection. Studies have shown that adiponectin inhibits TNF α production induced by ischemia–reperfusion, suggesting that adiponectin protects against inflammatory response to ischemia–reperfusion injury (Shibata *et al.* 2005, Siasos *et al.* 2012). We found that adiponectin deficiency resulted in markedly higher TNF α production in islets after hypoxia–reoxygenation. Otherwise, treatment with 100 μ g/kg adiponectin decreased the levels of TNF α . However, the suppressive effect of adiponectin was blocked by an anti-adiponectin MAB. Syngeneic islet transplantation revealed marked production of TNF α in islet grafts at 7 days post-transplant in adiponectin-deficient mice; however, TNF α levels in wild-type mice were suppressed.

Additionally, we found that adiponectin suppresses the production of TNF α stimulated by LPS in cultured islets. Our results indicate that the inhibitory action of adiponectin on the insular production of TNF α is dependent on the COX2–PGE₂–TNF α pathway. COX2 and the COX2 metabolite PGE₂ are reported to have important protective roles in islet grafts due to ischemia–reperfusion injury (Matsuda *et al.* 2005, Parazzoli *et al.* 2012). We found that adiponectin increased expression of COX2 in cultured islets stimulated by LPS or by hypoxia–reoxygenation and the release of PGE₂ from LPS-stimulated islets. However, the action of adiponectin on the increased expression of PGE₂ and suppressive production of TNF α was inhibited after administration of a COX2 inhibitor to LPS-stimulated islets. These data suggest that adiponectin suppresses LPS- and hypoxia–reoxygenation-induced secretion of TNF α through a COX2–PGE₂ pathway. If the protective actions of adiponectin on islet injury depends on the activity of the COX2–PGE₂–TNF α pathway, COX2 inhibition by adiponectin should cause an increase in apoptosis. However, abrogation of the adiponectin actions by inhibition of COX2 was not associated with the increase in apoptosis as in our previous study on cultured islets induced by LPS or hypoxia–reoxygenation. Presumably, the adiponectin-protective activity may

depend on additional pathways, which may include the inhibition of downstream pathways of TNF α .

NF- κ B is known to play a central role in the regulation of inflammatory reactions by NF- κ B. It induces the phosphorylation of the I κ B kinase complex, leading to I κ B phosphorylation and subsequent NF- κ B activation (Malinin *et al.* 1997, Tak & Firestein 2001). Activated NF- κ B has been observed in islet ischemic injury (Liuwantara *et al.* 2006, Negi *et al.* 2012, Porras *et al.* 2012). Additionally, TNF α induces NF- κ B activation resulting in islet graft destruction (Chang *et al.* 2003). Whether adiponectin prevents islet ischemic injury or inhibits the TNF α –NF- κ B pathway, our results show that adiponectin treatment significantly decreased the amount of NF- κ B (p65) DNA-binding complexes and suppressed I κ B- α phosphorylation induced by TNF α stimulation and hypoxia–reoxygenation. Additionally, the suppressive effect of adiponectin on NF- κ B activation was inhibited by an anti-adiponectin MAB. TNF α induces the activation of NF- κ B and induces the activation of the JNK or Akt pathways. We found that adiponectin had no effect on the phosphorylation of JNK or Akt kinase by TNF α stimulation. This suggests that adiponectin specifically suppressed the TNF α -induced activation of NF- κ B in islets. But when the suppressive effect of adiponectin on I κ B- α phosphorylation was abolished by the inhibition of I κ B- α phosphorylation, we unexpectedly found that apoptosis was increased and associated with a decreased expression of COX2 in cultured islets induced by TNF α . (Supplementary Figure 3, see section on supplementary data given at the end of this article). We speculate that the NF- κ B inhibitor could affect COX2 expression in islets treated with adiponectin.

In summary, adiponectin protects the islet from injury in response to ischemia–reperfusion. Adiponectin protection depends on the suppression of the islet production of TNF α through COX2 and the inhibition of TNF α -induced NF- κ B activation pathways. Islets are exceptionally susceptible to ischemia–reperfusion injury, an increased incidence of primary graft nonfunctionality, and β -cell death during a transplant procedure. Therefore, islets require protection during the early stages of the transplant procedure. Here, it is shown that adiponectin can improve islet tolerance of the process of ischemia–reperfusion and elevate islet transplantation efficiency, suggesting that the short-term administration of this factor may have practical clinical utility. Most significantly, we present the first direct evidence that adiponectin protects against islet ischemia–reperfusion injury, at least in part by the suppression of TNF α production and inhibition of NF- κ B activation via unknown mechanisms.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0568>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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