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Hypoxia Increases β-Cell Death by Activating Pancreatic Stellate Cells within the Islet

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Background: Hypoxia can occur in pancreatic islets in type 2 diabetes mellitus. Pancreatic stellate cells (PSCs) are activated during hypoxia. Here we aimed to investigate whether PSCs within the islet are also activated in hypoxia, causing β-cell injury.
Methods: Islet and primary PSCs were isolated from Sprague Dawley rats, and cultured in normoxia (21% O_2) or hypoxia (1% O_2). The expression of α-smooth muscle actin (α-SMA), as measured by immunostaining and Western blotting, was used as a marker of PSC activation. Conditioned media (hypoxia-CM) were obtained from PSCs cultured in hypoxia.
Results: Islets and PSCs cultured in hypoxia exhibited higher expressions of α-SMA than did those cultured in normoxia. Hypoxia increased the production of reactive oxygen species. The addition of N-acetyl-L-cysteine, an antioxidant, attenuated the hypoxia-induced PSC activation in islets and PSCs. Islets cultured in hypoxia-CM showed a decrease in cell viability and an increase in apoptosis.
Conclusion: PSCs within the islet are activated in hypoxia through oxidative stress and promote islet cell death, suggesting that hypoxia-induced PSC activation may contribute to β-cell loss in type 2 diabetes mellitus.

Keywords: Hypoxia; Insulin-secreting cells; Islets of Langerhans; Oxidative stress; Pancreatic stellate cells

INTRODUCTION

Pancreatic β-cell failure caused by β-cell dysfunction or loss plays an important role in the progression of type 2 diabetes mellitus (T2DM). The mechanisms underlying the process include oxidative stress, inflammation, endoplasmic reticulum stress, amyloid aggregation, islet fibrosis, and dedifferentiation [1]. Because the interventions currently available do not prevent the progression of T2DM, additional studies are necessary for a better understanding of the pathogenesis of β-cell failure.

The exposure of pancreatic islets to hypoxia may be another mechanism leading to β-cell failure in long-standing T2DM. Moreover, hypoxia in islet grafts is associated with β-cell death after islet transplantation [2]. Previous studies suggest that β-cells can become hypoxic because of the high oxygen consumption during insulin secretion, especially when the oxygen supply is not sufficient [3,4]. Recently, Bensellam et al. [5] re-

ported that hypoxia inhibited the adaptive unfolded protein response and endoplasmic reticulum-to-Golgi protein trafficking in β-cells with increased β-cell death. In addition, a compensatory increase in β-cell mass may compromise for the vascular density to maintain sufficient perfusion or oxygen delivery to the islet [6]. Abnormal islet vasculature including capillary loss and pancreatic arteriolosclerosis was also observed in T2DM [7,8]. Moreover, hypoxia induces oxidative stress [9], thus possibly aggravating hyperglycemia-induced oxidative stress.

Interestingly, hypoxia is known to activate pancreatic stellate cells (PSCs) [10,11]. PSCs play a crucial role in the pathogenesis of chronic pancreatitis and pancreatic cancer [12]. When PSCs transform from a quiescent state into an activated state, they exhibit a myofibroblastic phenotype, express α-smooth muscle actin (α-SMA) and produce collagen and other extracellular matrix proteins. The persistent activation of PSCs leads to fibrosis, which is associated with chronic pancreatitis and

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pancreatic cancer [13,14]. Interestingly, we and others have demonstrated that PSCs are also present within the islet and are activated in high glucose conditions and animal models of T2DM [15-21]. Furthermore, Zha et al. [22] isolated PSC-like cells from cultured rat islets and termed them islet stellate cells (ISCs). PSC activation in the islet may damage β-cells indirectly by promoting islet fibrosis, which may accelerate β-cell destruction or induce the disruption of β-cell connectivity [23-25]. Moreover, activated PSCs may damage β-cells directly by diminishing insulin secretion and inducing apoptosis [26-28].

Therefore, exposure of the islet to hypoxia might induce the activation of intra-islet PSCs, which in turn would damage β-cells leading to the progressive β-cell failure observed in T2DM. However, few studies have addressed this possibility. Here, we examined the hypoxia-induced activation of PSCs and its underlying mechanism, as well as the detrimental effect of activated PSCs on β-cell viability.

METHODS

Isolation and culture of rat islets and primary PSCs

Islets were isolated from 7-week-old Sprague Dawley (SD) rats and cultured in RPMI 1640 medium containing 11.1 mM glucose and 10% FBS. Primary PSCs were isolated from 14-week-old SD rats as reported previously [20,21]. PSCs were cultured in DMEM/Ham's F-12 medium (1:1) containing 17.5 mM glucose and 16% fetal calf serum. The experiments were performed using PSCs at passages 1 to 6 after isolation, except for those that required freshly isolated PSCs. PSCs and islets were incubated in normoxia (20% O_2) or hypoxia (1% O_2) using a hypoxic chamber (Galaxy 14S; Eppendorf, Hamburg, Germany). The experimental protocol was approved by the Institutional Animal Care and Use Committee in School of Medicine, The Catholic University of Korea (CUMC-2018-0209-03).

Immunohistochemistry

Pimonidazole staining was performed to detect decreased cellular oxygen tension in islets using a commercial kit (Hypoxyprobe Kit; Hypoxyprobe, Burlington, MA, USA) with 10 μM pimonidazole for 2 hours in normoxia or hypoxia.

α-SMA staining was performed to detect activated PSCs among primary PSCs and islets, as α-SMA expression is the most commonly used index of PSC activation [13]. Primary PSCs were cultured on cover glasses coated with poly-L-lysine. The cells were fixed in 4% paraformaldehyde and incubated overnight at 4°C with a mouse anti-α-SMA antibody (1:400; Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated with a rhodamine-labeled anti-mouse IgG antibody (1:100) as the secondary antibody. The nuclei were stained with 4',6-diamidino-2-phenyl-indole (DAPI). The percentage of α-SMA-positive cells among primary PSCs was calculated. Islets were fixed in 4% paraformaldehyde, and pelleted in 2.5% agar (US Biological, Salem, MA, USA). The specimens were embedded in paraffin, and cut into 3 μm. The sections were incubated overnight at 4°C with the mouse anti-α-SMA-antibody (1:400; Sigma-Aldrich), as the primary antibody followed by incubation with a biotin-conjugated rabbit anti-mouse IgG and avidin-biotin complex (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA, USA), as the secondary antibody. The slides were developed using diaminobenzidine (Sigma-Aldrich) and counterstained with hematoxylin. The percentage of the α-SMA-stained area per islet section was calculated.

Western blot analysis

Western blot analyses were performed as described previously [15]. The primary antibodies used were the anti-α-SMA antibody (1:2,000; Sigma-Aldrich) and anti-β-actin antibody (1:10,000; Abcam, Cambridge, MA, USA).

Measurement of reactive oxygen species

To measure the production of measurement of reactive oxygen species (ROS), PSCs were loaded with 10 μM dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA) for 30 minutes at 37°C. The cells were analyzed using a luminometer (excitation/emission at λ=490/535 nm) in a black 96-well plate. A fluorescence microscope was used to observe DCF fluorescence.

Preparation of conditioned media from PSCs

Primary PSCs at passage 1 to 3 after isolation were grown to 80% confluence, then cultured for 48 hours in normoxia or hypoxia. The media were collected, centrifuged to remove cells, and stored at –20°C until use. For control experiments, conditioned media (CM) from C2C12 cells (a mouse myoblast cell line) were prepared in the same way.

Cell viability and apoptosis assay

Rat islets were incubated for 48 hours in media for culturing PSCs (PSC media), CM from PSCs in normoxia (normoxia-CM) and hypoxia (hypoxia-CM), and CM from C2C12 cells in

normoxia and hypoxia. Cell viability was determined using acridine orange (AO)/propidium iodide (PI) staining. Islets were visualized using a fluorescence microscope. Digitalized images were captured and the areas that were stained with PI were quantified using Adobe Photoshop CS 8.0 (Adobe Systems Incorporated, San Jose, CA, USA). In addition, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to detect apoptosis in islets, as described previously [29]. Islets were fixed with 4% paraformaldehyde, pelleted in 2.5% agar, and embedded in paraffin. The *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) was used. Apoptotic cells (TUNEL-positive cells) were quantified using a fluorescence microscope. Nuclei were stained with DAPI.

Detection of various cytokines released from PSCs

Various cytokines, including the tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ), were measured in PSC media and normoxia-CM and hypoxia-CM from PSCs using a multiplex enzyme-linked immunosorbent assay (ELISA) kit (Rat Inflammatory Cytokines Multi-Analyte ELISArray Kit; Qiagen, Hilden, Germany) and individual ELISA kits (Abcam).

Statistical analysis

Data are expressed as the mean±standard error of the mean (SEM). Differences between groups were evaluated using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA, USA). To analyze the quantitative variables between groups, Student's *t*-test or analysis of variance (ANOVA) with *post hoc* test for multiple comparisons was used. *P* values of <0.05 were considered to show significance.

## RESULTS

Activation of PSCs within the islet after exposure of hypoxia

First, we performed pimonidazole staining in islets. We detected pimonidazole adducts in islets cultured in 1% hypoxia, but not in 20% normoxia, thus confirming the presence of cellular hypoxia under this condition (Fig. 1A). Next, we examined the
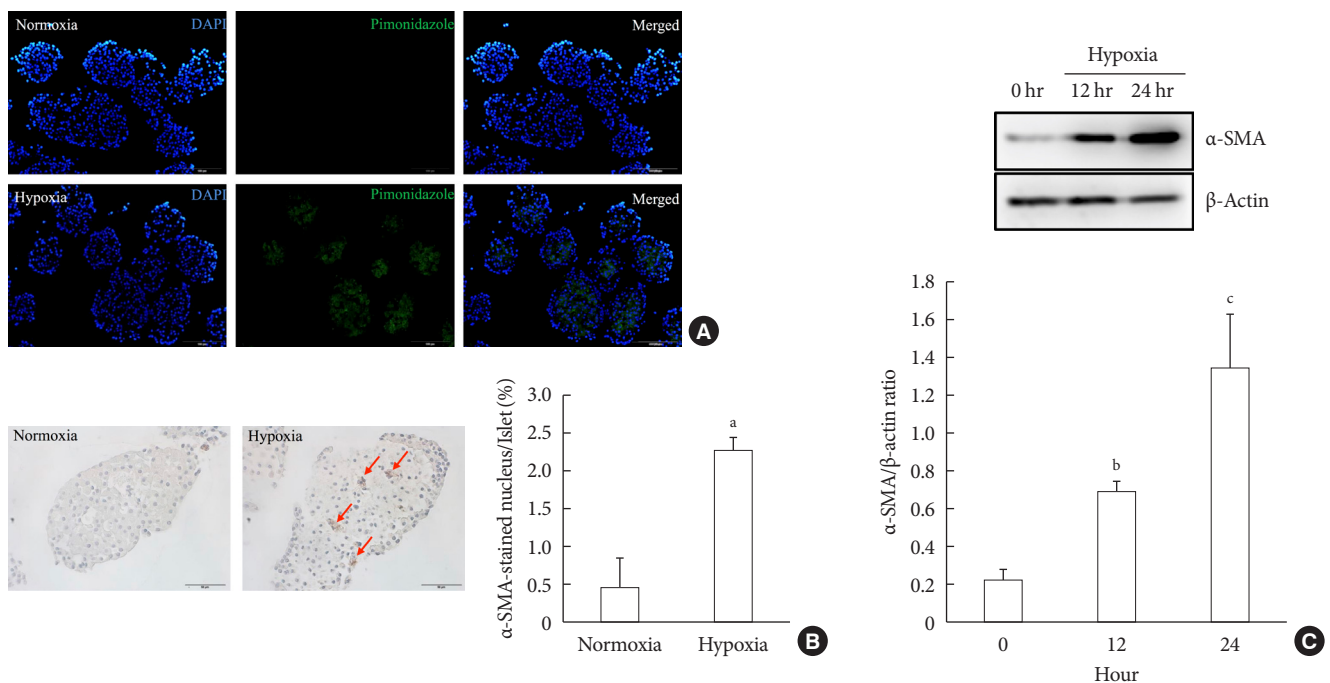
Fig. 1. Activation of pancreatic stellate cells (PSCs) in islets after hypoxia. (A) Images showing pimonidazole (green) staining of islets after incubation in normoxia or hypoxia for 2 hours. The nuclei were stained with 4′,6-diamidino-2-phenyl-indole (DAPI, blue). (B) Images showing α-smooth muscle actin (α-SMA) staining and the percentage of α-SMA-positive cells within the islet after incubation in normoxia or hypoxia for 12 hours. Arrows indicate cells expressing α-SMA (brown). Bar, 100 μm. (C) The expression of α-SMA in Western blot analysis. Values are mean±standard error of the mean (*n*=3). [a]*P*<0.05 for normoxia vs. hypoxia, [b]*P*<0.01 for 12 hours vs. 24 hours, [c]*P*<0.01 for 0 hour vs. 24 hours.
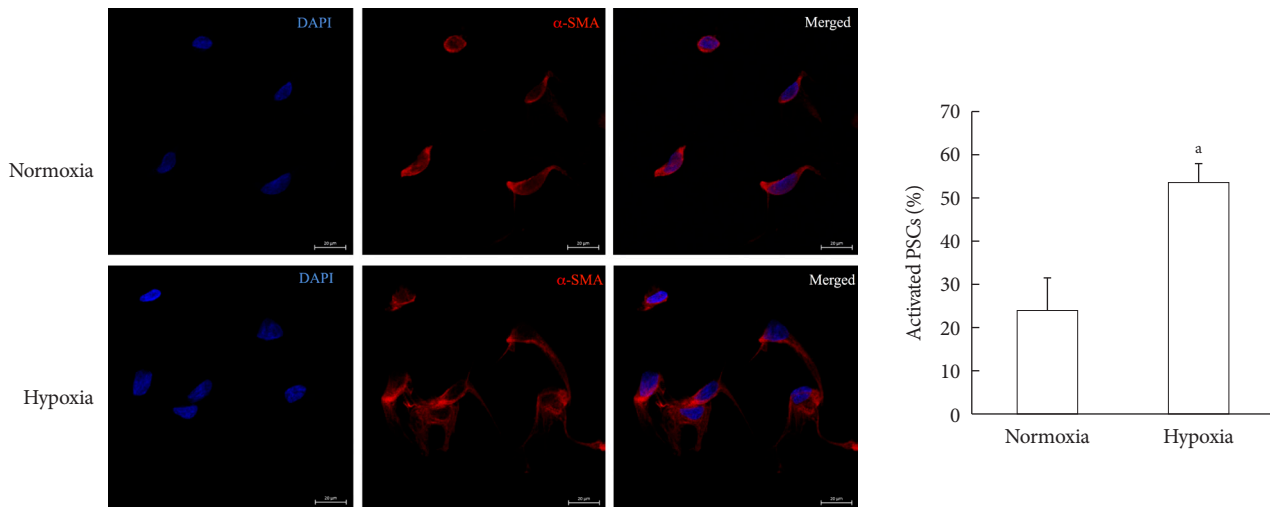
Fig. 2. Activation of pancreatic stellate cells (PSCs) after hypoxia among freshly isolated PSCs. Images showing α-smooth muscle actin (α-SMA) staining and the percentage of activated PSCs after the cells were incubated in hypoxia or normoxia for 48 hours. Bar, 20 μm. Values are presented as mean±standard error of the mean ($n=4$). DAPI, 4´,6-diamidino-2-phenyl-indole. [a]$P<0.05$ for normoxia vs. hypoxia.

presence of PSC activation in islets after exposure to hypoxia or normoxia for up to 24 hours. Immunostaining showed the existence of multiple α-SMA-positive cells in islets cultured in hypoxia, indicating the presence of activated PSCs. The percentage of α-SMA-positive cells in islets cultured in hypoxia was significantly higher than that detected in islets cultured in normoxia ($2.25\%\pm0.19\%$ vs. $0.47\%\pm0.39\%$; $P<0.05$) (Fig. 1B). Western blot analysis also showed a significant increase in the expression of α-SMA in islets cultured in hypoxia (Fig. 1C).

Activation of primary PSCs after exposure to hypoxia

Freshly isolated PSCs were incubated in hypoxia or normoxia for 48 hours. α-SMA staining showed that most PSCs exposed to hypoxia transformed into a myofibroblast-like phenotype, whereas most PSCs exposed to normoxia remained quiescent (Fig. 2).

Involvement of oxidative stress in PSC activation after exposure of hypoxia

To measure ROS generation, primary PSCs were exposed to DCF, a fluorescent marker of cellular oxidant production. Fig. 3A shows that exposure to hypoxia for 48 hours induced a higher fluorescence intensity compared with normoxia, which indicated the induction of oxidative stress by hypoxia. To investigate whether the hypoxia-induced facilitation of PSC activation was prevented by antioxidant treatment, we added 2.5 mM

N-acetyl-L-cysteine (NAC) to islets cultured in hypoxia for 24 hours. Western blot analysis showed a significant attenuation of the upregulation of a-SMA induced by hypoxia (Fig. 3B). In addition, α-SMA staining of primary PSCs cultured in hypoxia with or without 2.5 mM NAC for 48 hours confirmed that NAC treatment attenuated the activation of PSCs (Fig. 3C).

Effect of PSC activation on islet viability

To examine the effect of PSC activation on β-cell survival, we incubated islets for 48 hours in PSC media, normoxia-CM, and hypoxia-CM obtained from primary PSCs, and CM from C2C12 cells in normoxia and hypoxia. The AO/PI staining showed that islets incubated with hypoxia-CM from PSCs exhibited increased cell death compared with those incubated with PSC media (PI-positive cells: $22.45\%\pm4.71\%$ vs. $2.84\%\pm1.35\%$, $P<0.05$) (Fig. 4A). In contrast, CM from C2C12 cells did not affect islet survival (Fig. 4B). In addition, the TUNEL assay showed that islets incubated with hypoxia-CM from PSCs contained more TUNEL-positive cells ($38.32\%\pm1.70\%$) than did those incubated with normoxia-CM ($21.50\%\pm1.75\%$) or PSC media ($9.77\%\pm1.50\%$) (all $P<0.05$) (Fig. 4C).

Measurement of various cytokines in conditioned media from PSCs

To examine whether cytokines released from PSCs induced islet cell death, we measured the levels of various cytokines in
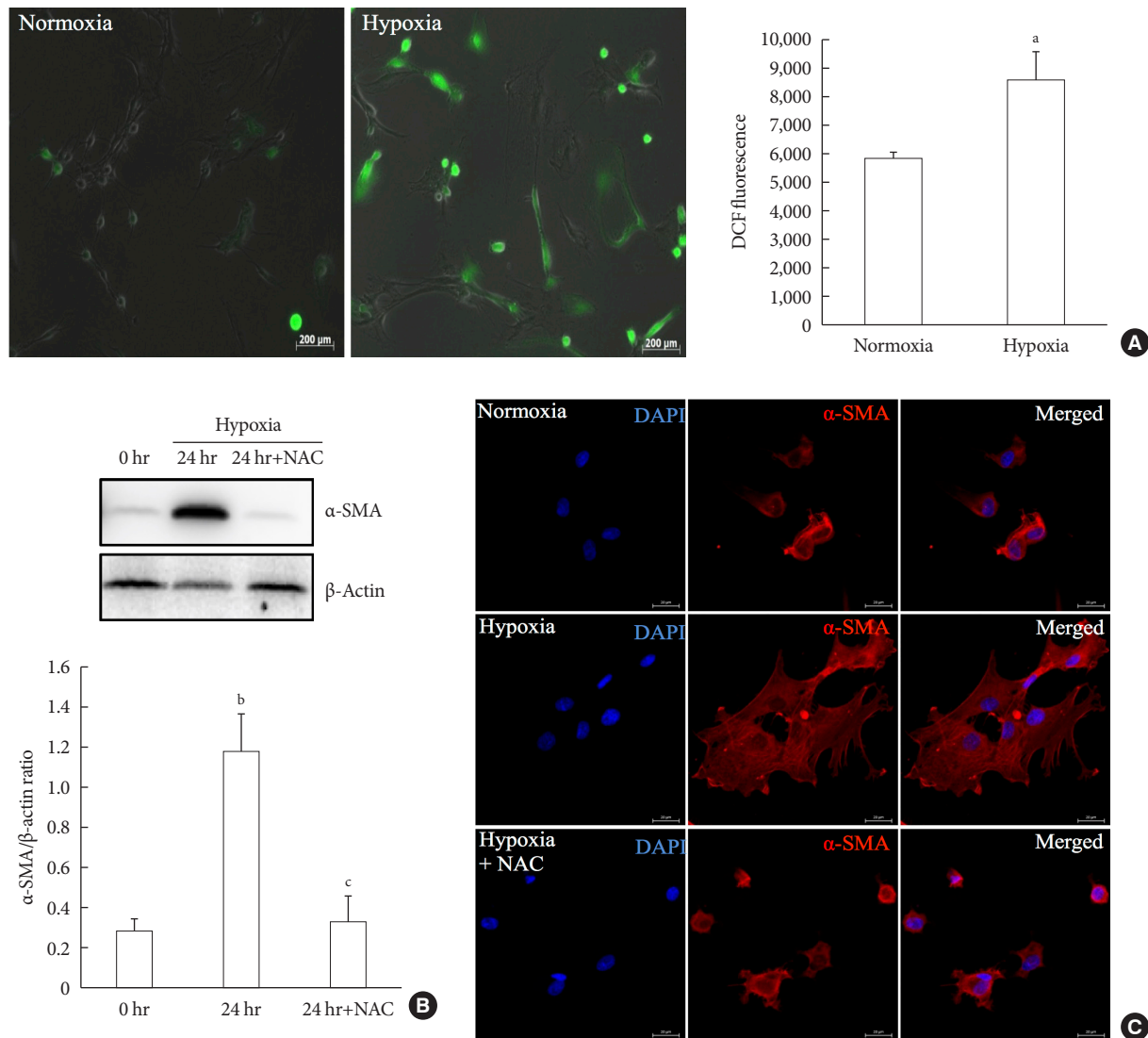
Fig. 3. Involvement of oxidative stress in pancreatic stellate cell (PSC) activation after hypoxia. (A) Generation of reactive oxygen species. Images showing dichlorodihydrofluorescein diacetate (DCF) fluorescence (green) in PSCs after exposure to hypoxia or normoxia for 48 hours. DCF fluorescence was quantified using a scanning fluorometer. Bar, 200 μm. Values are presented as mean±standard error of the mean ($n=6$). (B) Western blot analysis showing the expression of α-smooth muscle actin (α-SMA) in islets after hypoxia in the presence or absence of treatment with 2.5 mM N-acetyl-L-cysteine (NAC). Values are presented as mean±standard error of the mean ($n=3$). (C) Representative images of α-SMA staining in primary PSCs cultured in hypoxia with or without 2.5 mM NAC for 48 hours. Bar, 20 μm. DAPI, 4′,6-diamidino-2-phenyl-indole. [a]$P<0.05$ for normoxia vs. hypoxia, [b]$P<0.01$ for 0 hr (0 hour) vs. 24 hr (24 hours), [c]$P<0.05$ for 24 hours vs. 24 hours+NAC.

PSC media, normoxia-CM, and hypoxia-CM from PSCs using two different ELISA kits. However, none of these cytokines were detected (Tables 1 and 2).

DISCUSSION

We demonstrated for the first time that hypoxia activates PSCs

within the islet by inducing oxidative stress. We further showed that hypoxia-activated PSCs increase β-cell death via apoptosis.

Hypoxia activates PSCs, which contribute to pancreatic cancer progression [10,11]. To our knowledge, however, no study has demonstrated that hypoxia activates PSCs present within the islet. To induce cellular hypoxia, we incubated rat islets in 1% hypoxic conditions. Detection of pimonidazole adducts in
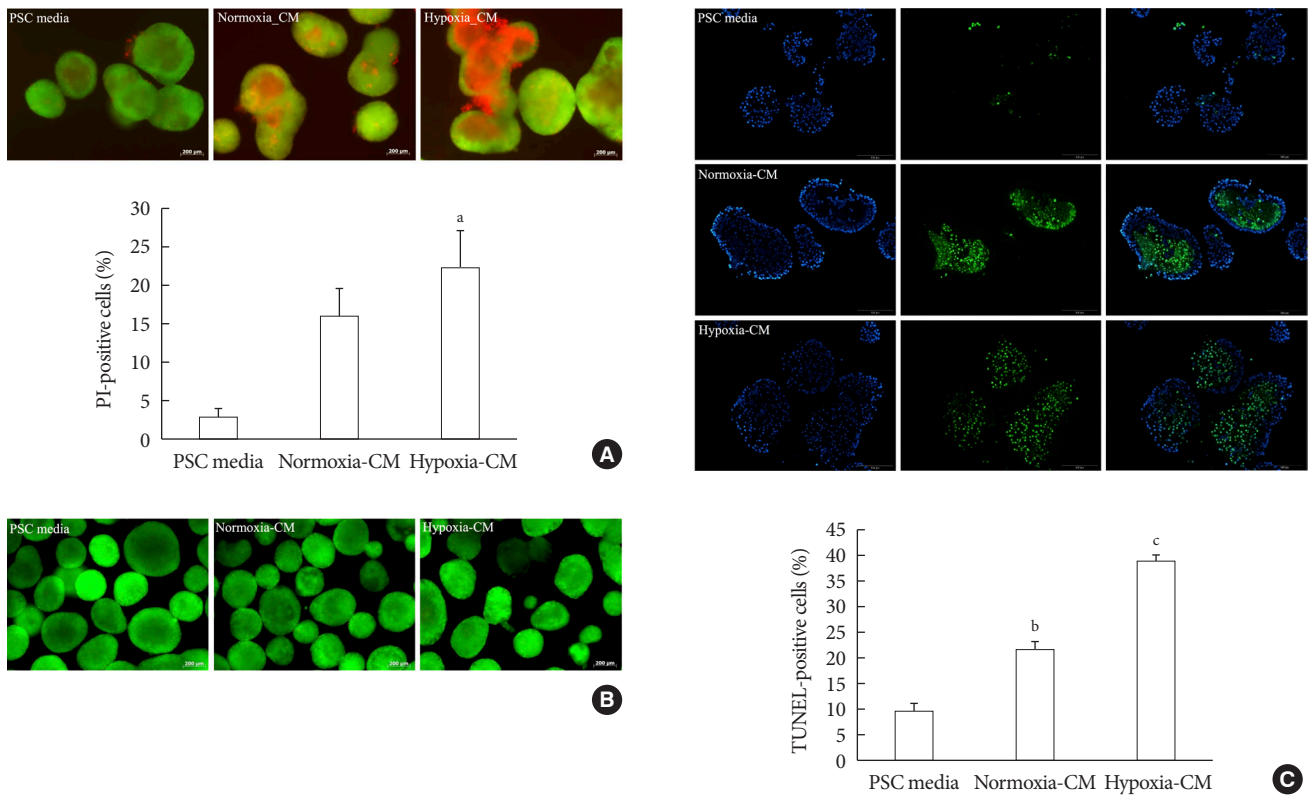
Fig. 4. Effect of pancreatic stellate cell (PSC) activation on islet viability. (A) Representative images of acridine orange (AO, green)/propidium iodide (PI, red) staining and quantification of PI-positive cells in rat islets incubated in PSC media and normoxia-conditioned media (CM) and hypoxia-CM from PSCs for 48 hours. Bar, 200 μm. (B) Representative images of AO/PI staining in rat islets incubated in PSC media and normoxia-CM and hypoxia-CM from C2C12 cells for 48 hours. Bar, 200 μm. (C) Representative images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and quantification of TUNEL-positive cells in rat islets incubated in PSC media and normoxia-CM and hypoxia-CM from PSCs for 48 hours. The nuclei were stained with 4′,6-diamidino-2-phenyl-indole (DAPI, blue). Bar, 100 μm. Values are presented as mean ± standard error of the mean ($n = 3$). [a]$P < 0.05$ for PSC media vs. hypoxia-CM, [b]$P < 0.05$ for PSC media vs. normoxia-CM, [c]$P < 0.05$ for PSC media vs. hypoxia-CM and for normoxia-CM vs. hypoxia-CM.

Table 1. Measurement of various cytokines in conditioned media from PSCs using a multiplex ELISA kit

Samples	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12	IL-13	IFN-γ	TNF-α	GM-CSF	RANTES
Negative control	0.196	0.467	0.555	0.112	0.506	1.170	0.131	0.195	0.920	0.615	0.139	0.125
PSC media (sample 1)	0.197	0.436	0.519	0.101	0.422	1.067	0.118	0.163	0.857	0.558	0.105	0.118
Normoxia-CM (sample 1)	0.180	0.417	0.488	0.099	0.393	1.014	0.112	0.152	0.813	0.505	0.109	0.120
Hypoxia-CM (sample 1)	0.170	0.415	0.515	0.172	0.388	0.968	0.108	0.157	0.778	0.502	0.121	0.112
PSC media (sample 2)	0.171	0.421	0.506	0.102	0.409	1.042	0.119	0.152	0.829	0.501	0.085	0.094
Normoxia-CM (sample 2)	0.180	0.418	0.494	0.097	0.403	1.018	0.117	0.156	0.79	0.503	0.087	0.102
Hypoxia-CM (sample 2)	0.162	0.426	0.495	0.091	0.412	1.065	0.105	0.158	0.813	0.482	0.086	0.105
Positive control	3.646	Overflow	Overflow	0.812	0.852	3.603	2.806	Overflow	Overflow	Overflow	2.969	Overflow

Values are presented as absorbance values at 450 nm.

PSC, pancreatic stellate cell; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte macrophage-colony stimulating factor; RANTES, regulated on activation normal T cell expressed and secreted; CM, conditioned media.

Table 2. Measurement of IL-1β, IFN-γ, and TNF-α in conditioned media from PSCs using individual ELISA kits

Samples	IFN-γ	IL-1β	TNF-α
PSC media (sample 1)	ND	ND	ND
Normoxia-CM (sample 1)	ND	ND	ND
Hypoxia-CM (sample 1)	ND	ND	ND
PSC media (sample 2)	ND	ND	ND
Normoxia-CM (sample 2)	ND	ND	ND
Hypoxia-CM (sample 2)	ND	ND	ND

The detection limit of cytokines: IFN-γ, 1.1 pg/mL; IL-1β, 80 pg/mL; TNF-α, 25 pg/mL.

IL-1β, interleukin-1β; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; PSC, pancreatic stellate cell; ELISA, enzyme-linked immunosorbent assay; ND, not detected; CM, conditioned media.

islets indicates the presence of severe hypoxia [30]. After hypoxic exposure, an increased number of α-SMA-positive cells was observed in islets, together with an increase in the expression of the α-SMA protein, as measured by Western blot analysis. We also isolated primary rat PSCs and incubated them in hypoxic conditions. Consistent with previous reports [10,11], most PSCs exposed to hypoxia became activated.

Oxidative stress plays an important role in PSC activation [31,32]. Hypoxia induces oxidative stress in PSCs [10,11], as oxygen depletion stimulates mitochondria to produce ROS [33,34]. Here, we found that ROS production was increased in PSCs during hypoxia. Co-treatment with NAC, an antioxidant, prevented the hypoxia-induced facilitation of PSC activation in islets and primary PSCs. These findings are consistent with a previous study reported by Lei et al. [11], which showed the suppressive effect of α-mangostin (an antioxidant) on hypoxia-induced PSC activation.

Previous studies that were performed using cocultures of PSCs with RIN-5F cells or rat islets [26,28] and treatment of INS-1 cells with CM from PSCs or ISCs [27,35] demonstrated that activated PSCs could damage β-cells by reducing insulin secretion or inducing cell death and apoptosis. In this study, we measured cell death and apoptosis in cultured islets using AO/PI staining and the TUNEL assay, respectively. We found that islet cell death and apoptosis were more frequent after incubation with hypoxia-CM from PSCs compared with PSC media. This finding was specific to PSCs because CM from C2C12 cells did not affect islet survival. Our results suggest that mediators produced by PSCs induce cell death and apoptosis directly in β-cells. Therefore, we measured the levels of various cyto-

kines, including TNF-α, IL-1β, or IFN-γ, which are known to induce apoptosis in β-cells [36], in CM from PSCs. However, none of these cytokines were detected.

The current study had several limitations. First, we cultured the cells under severe hypoxic conditions. Second, we did not use ISCs isolated from the islet [22] in our experiment, but the characteristics of ISCs are similar to those of typical PSCs regarding biomarkers and activation [37]. Third, we were not able to detect cytokines in CM from PSCs, contrary to the findings of a previous study reported by Li et al. [35]. The reason for this discrepancy is not clear. Factors other than cytokines such as lipid substances, microRNAs, or exosomes could be more important in PSC activation-induced β-cell death. Exosomes are membrane-enclosed nanovesicles containing diverse bioactive molecules including lipids, proteins and microRNAs. It has been recognized that extracellular vesicles involving exosomes are significant mediators of communications between cells including PSCs [37]. Therefore, it is necessary to compare the exosomal cargo between the exosomes from the normoxia-CM and hypoxia-CM or between the exosomes from primary PSCs and C2C12 cells in the future.

In conclusion, our data suggest that PSCs within the islet are activated in hypoxia through oxidative stress and promote β-cell apoptosis, suggesting that hypoxia-induced PSC activation contributes to β-cell loss. Therefore, therapies targeting PSC activation might be beneficial for the prevention of β-cell failure in T2DM and islet transplantation. However, additional studies are needed to elucidate the precise mechanism underlying the induction of β-cell death by PSC activation.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: E.L., G.R.R., K.H.S.
Acquisition, analysis, or interpretation of data: J.J.K., E.L., G.R.R., S.H.K., Y.B.A., K.H.S.
Drafting the work or revising: J.J.K., K.H.S.
Final approval of the manuscript: J.J.K., E.L., G.R.R., S.H.K., Y.B.A., K.H.S.

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