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Targeting Nrf2 by dihydro-CDDO-trifluoroethyl amide enhances autophagic clearance and viability of β-cells in a setting of oxidative stress

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ABSTRACT

Nrf2 appears to be a critical regulator of diabetes in rodents. However, the underlying mechanisms as well as the clinical relevance of the Nrf2 signaling in human diabetes remain to be fully understood. Herein, we report that islet expression of Nrf2 is upregulated at an earlier stage of diabetes in both human and mice. Activation of Nrf2 suppresses oxidative stress and oxidative stress-induced β-cell apoptosis while enhancing autophagic clearance in isolated rat islets. Additionally, oxidative stress per se activated autophagy in β-cells. Thus, these results reveal that Nrf2 drives a novel antioxidant independent autophagic clearance for β-cell protection in the setting of diabetes.
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1. Introduction

Diabetes is a major worldwide public health problem with an increasing prevalence and mortality. It is predicted that approximately 300 million people will be diabetic by 2025 [1]. Of further concern is the fact that the underlying mechanism is far from clear and the existing therapy is not satisfactory.

In general, type I diabetes is characterized by chronic hyperglycemia resulting from absolute deficiency of pancreatic insulin secretion due to autoimmune-mediated destruction of pancreatic β-cell islets; whereas type II diabetes is characterized by chronic hyperglycemia due to lack of pancreatic insulin deficiency and/or peripheral insulin resistance. That is, in a type II pre-diabetic state, pancreatic β-cells overproduce insulin to compensate for insulin resistance but eventually these cells decompensate and the clinical manifestations of diabetes become apparent [2]. Nevertheless, progressively decreased pancreatic β-cell function and β-cell mass are common features of subjects with type I and type II diabetes.

Because of the high secretory activity, β-cells are constantly exposed to various kinds of stresses, such as glucolipotoxicity and oxidative stress [3,4]. In diabetes, oxidative stress is a consequence of high circulating glucose levels; however, chronic oxidative stress also causes β-cell death [3,4]. It is worthy to note that compared with other cell types, the expression of antioxidant enzymes, such as catalase and glutathione peroxidase in β-cells, is very low [3]. Thus, β-cells are extremely sensitive to oxidative stress which is a major contributor to β-cell dysfunction. Recently, growing evidence has indicated that nuclear factor erythroid 2-related factor 2 (Nrf2), a master transcriptional factor for induction of a spectrum of cytoprotective phase II enzymes and antioxidant proteins, may be a critical negative regulator to the onset of diabetes via its abilities to suppress oxidative stress as well as to interact with other transcription factors and receptors implicated in

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Table 1
Clinical data of the male patients with and without diabetes.

	Non-diabetes (*n*)	Diabetes (*n*)	*P* value
Male			
Age (years)	56.5 ± 6.1 (3)	56.2 ± 3.9 (3)	>0.05
FPG (mmol/L)	5.52 ± 0.18 (3)	6.99 ± 0.64 (3)	<0.05
Female			
Age (years)	58.6 ± 8.5 (3)	54.3 ± 4.1 (3)	>0.05
FPG (mmol/L)	5.49 ± 0.23 (3)	7.33 ± 0.37 (3)	<0.05

FPG, fasting plasma glucose.

Table 2
STZ-induced diabetes in mice.

	Vehicle		STZ	
(day)	0	12	0	12
(*n*)	(5)	(5)	(5)	(5)
Body weight (g)	22.3 ± 1.3	23.9 ± 0.9	22.2 ± 1.3	23.5 ± 2.1
Blood glucose (mmol/L)	7.3 ± 0.4	7.6 ± 0.3	7.2 ± 0.3	21.4 ± 1.1*

* $P < 0.05$ vs. Vehicle or STZ untreated groups.

metabolic regulation [5]. Moreover, the magnitude of Nrf2 activation seems to be functionally relevant in specific settings. For example, genetic induction of Nrf2 in leptin-deficient (ob/ob) mice worsens insulin resistance and impairs adipogenesis [6]; whereas the same genetic Nrf2 induction as well as oral administration of Nrf2 inducer CDDO-Im {oleanolic acid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole}, a synthetic derivative of triterpenoid, in leptin receptor deficient (db/db) mice prevents the onset of diabetes with a remarkable preservation of β-cell mass in pancreata [7]. Although the precise reasons for these discrepancies are unclear, the notion that Nrf2 protects pancreatic β-cells against reactive oxidative species (ROS)-mediated damage [8–11] has been recently demonstrated utilizing β-cell specific Nrf2 gain- and loss-of-function approaches [12]. Nevertheless, other potentially ROS-independent mechanisms by which Nrf2 preserves β-cell mass remain to be determined.

It has been demonstrated that macroautophagy (commonly known as autophagy), an evolutionarily conserved mechanism for bulk degradation of cytoplasmic components, plays a critical role not only in the maintenance of normal islet architecture and function but also in the adaptive response of β-cells in diabetic settings such as insulin resistance and oxidative stress [13,14]. Autophagy begins with formation of the autophagosome, a double-membrane structure of unknown origin that engulfs cytoplasmic contents and then fuses them with lysosome to form autolysosome (known as autophagosome mature) whereupon proteolysis of the engulfed materials occurs [15]. Of note, autophagic

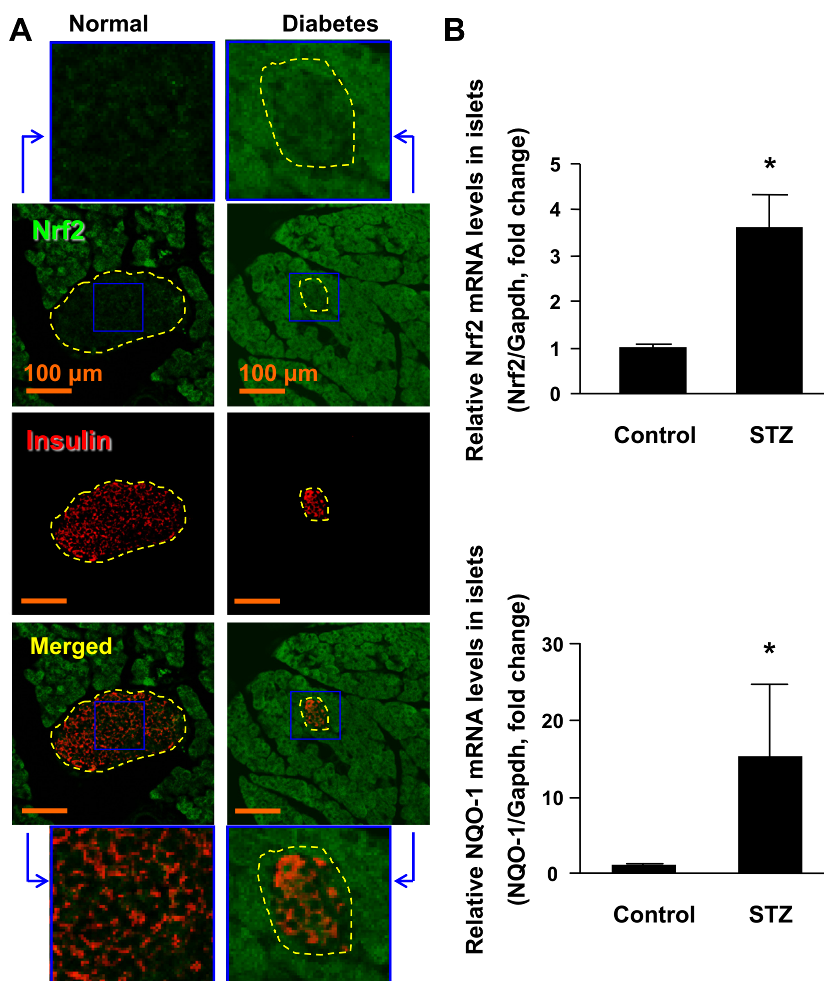

Fig. 1. Expression of Nrf2 in diabetic islets. (A) Representative immunofluorescent staining of Nrf2 in islets from 3 normal and diabetic mice 12 days post-injection of STZ. Nrf2, green; insulin, red. (B) Q-PCR analysis of mRNA expression of Nrf2 and NQO-1 in islets from normal and diabetic mice 12 days post-injection of STZ. $n = 5$, $^*P < 0.05$ vs. control.

clearance of the toxic ubiquitinated proteins in islets is likely a key mechanism to protect β-cells from cellular damage caused by oxidative stress associated with diabetes [13]. Importantly, emerging evidence has uncovered that Nrf2 mediates autophagic clearance of ubiquitinated proteins secondary to ROS formation in macrophages [16]. Given ROS are important mediators of autophagy activation and Nrf2 is an endogenous inhibitor of ROS formation in a variety of cell types [17,18], it is plausible that Nrf2 facilitate cellular autophagic clearance via a mechanism independent of ROS formation. While this hypothesis remains to be further explored, it is unclear whether activation of Nrf2 in the islets facilitates autophagic clearance to protect β-cells from oxidative stress-induced cellular damage in a diabetic setting.

In the present study, we explored the potential involvement of Nrf2 in human diabetes and the molecular mechanism of Nrf2-mediated islet survival utilizing isolated rat islets in a setting of oxidative stress. We found that Nrf2 is not only able to suppress oxidative stress but also can drive a novel antioxidant independent autophagic clearance of ubiquitinated toxic proteins in β-cells thereby improving β-cell survival and preventing islet injury in a setting of diabetes.

2. Materials and methods

Pancreatic tissue was obtained from non-diabetic controls and diabetic patients or mice at a relative early stage of diabetes (Tables 1 and 2, Supplementary Table 1 and Supplementary Fig. 1). Rat islets were isolated and cultured as previously described [19,20]. Islet function was assessed by monitoring the glucose-stimulated insulin secretion (GSIS) test. Rat islets cultured with 500 μl RPMI 1640 medium supplemented with 1% FBS were pretreated with or without a novel Nrf2 activator, dihydro-CDDO-trifluoroethyl amide (dh404) (300 nM) [21] for 6 h and followed with or without treatment of H_2O_2 (150 μM) and dh404 (300 nM) for additional 18 h. INS-1 cells, a β-cell line, were purchased from American Type Cell Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 10% FBS. Nrf2 knockdown was performed by double transfection of Nrf2 RNAi oligonucleotides. INS-1 cells cultured in serum free RPMI 1640 medium were pretreated with dh404 (300 nM) for 6 h and followed with or without treatment of H_2O_2 (100 μM) and dh404 (300 nM) for additional 18 h. Western blot, immunohistochemical and immunofluorescent staining were performed using appropriate antibodies

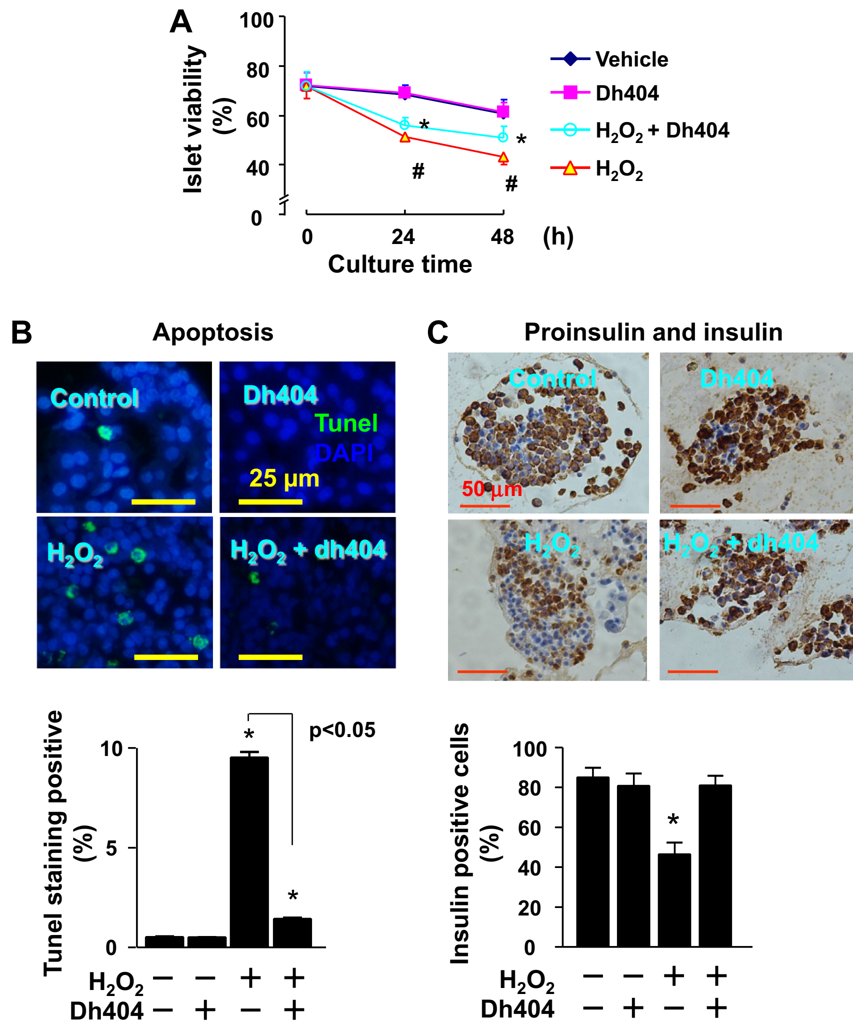

Fig. 2. Effect of dh404 on H_2O_2-induced islet injury and islet cell apoptosis. (A) Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 48 h. Viability of the islets was determined by a LDH cytotoxicity detection kit. $n = 3$, $^*P < 0.05$ vs. H_2O_2 group; $^\#P < 0.05$ vs. Vehicle or dh404 groups. (B) Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. The islets were then subjected to TUNEL staining. Apoptotic cells, green; Nuclei, blue. Percentage of positive staining was calculated based on the total number of counter-stained cells ($n = 3$). $^*P < 0.05$ vs. control (–). (C) Effect of dh404 on H_2O_2-induced decreases in the number of cells positive with pro-insulin and insulin staining in islets. Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. The islets were then subjected to proinsulin and insulin staining. Percentage of positive staining was calculated based on the total number of counter-stained cells ($n = 3$). $^*P < 0.05$ vs. other groups.

as previously described [21]. The methods are described in details in the Supplementary materials.

3. Results

3.1. Upregulation of Nrf2 in islets of early diabetes

The expression of Nrf2 in diabetic islets is poorly understood, thus we determined the expression of Nrf2 in islets in both human and mice at a relative early stage of diabetes. Immunofluorescent staining revealed that there was clear destruction of islets characterized by a smaller and irregular architecture in diabetic human and mice compared with the normal subjects (Supplementary Fig. 2 and Fig. 1A). The Nrf2 expression was relative low in normal islets compared with the surrounding tissues (Supplementary Fig. 2 and Fig. 1A), which is consistent with the previous findings of low expression levels of several Nrf2 target genes such as superoxide dismutase (SOD)1, SOD2, catalase and glutathione peroxidase in islets [3,17]. However, the Nrf2 expression was upregulated in the diabetic islets relative to the normal islets while it also was upregulated in non-islet cells of the diabetic pancreas compared with the normal control (Supplementary Fig. 2 and Fig. 1A). When we carefully analyzed the Nrf2 expression in normal and diabetic human islets, we observed the nuclear accumulation of Nrf2 in the diabetic islets but not in the normal control (Supplementary Fig. 2A and B), suggesting a potential activation

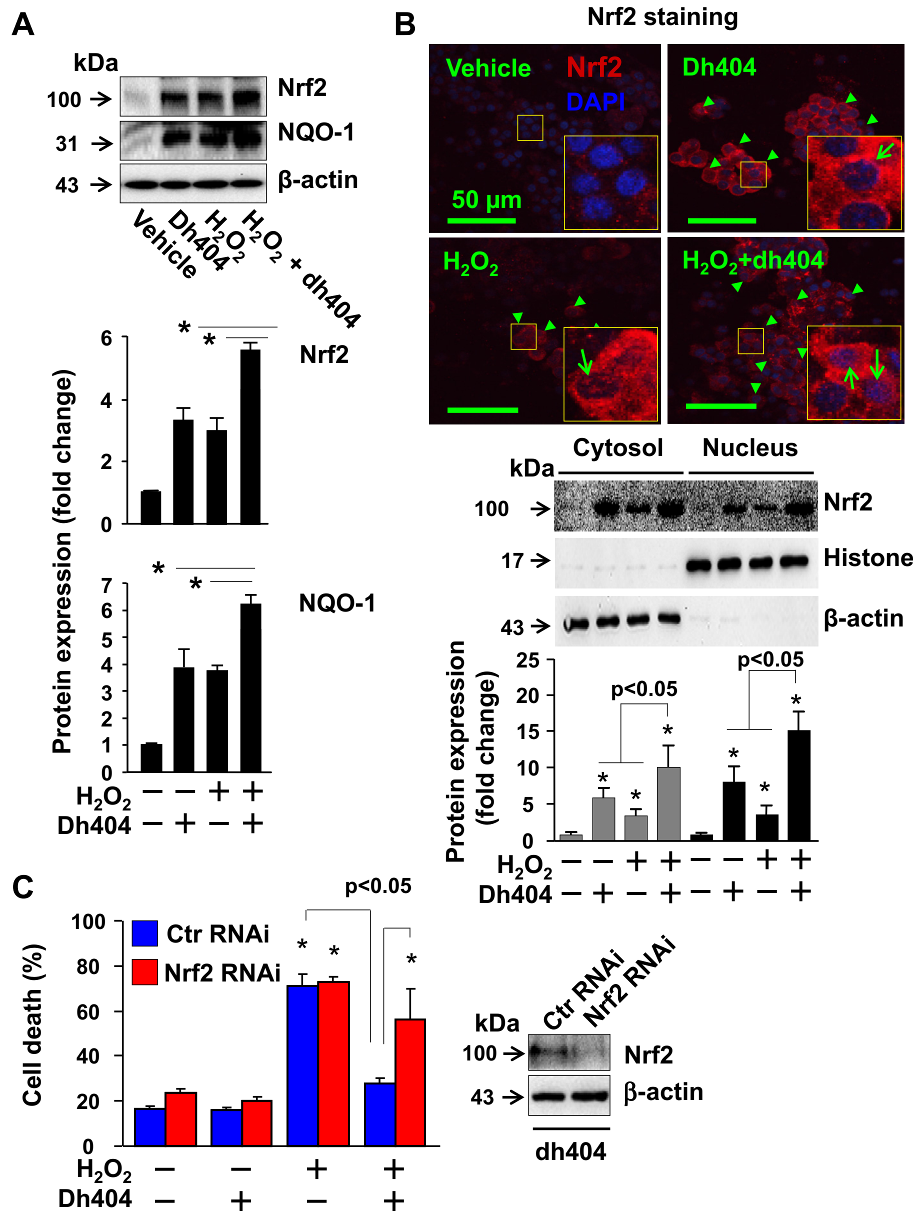

Fig. 3. Role of Nrf2 in dh404-induced protection against H_2O_2-mediated β-cell death. (A and B) Nrf2 activation. Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. Upper left panel: representatives of immunoblots for whole cellular Nrf2 and NQO-1. Lower left panel: semi-quantified densitometric analysis. $n = 3$, $^*P < 0.05$. Upper right panel: representatives of Nrf2 staining. Nrf2 (red), Nuclei (blue). Lower right panel: representatives of immunoblots of cytosolic and nuclear Nrf2, as well as densitometric analysis. $n = 4$, $^*P < 0.05$ vs. control (−) in the same group. (C) Effect of Nrf2 RNAi on dh404-induced cytoprotection in INS-1 β-cells in a setting of oxidative stress. INS-1 cells transfected with control scramble oligonucleotides (Ctr RNAi) or Nrf2 RNAi oligonucleotides were treated with or without H_2O_2 (100 μM) and/or dh404 (300 nM) as indicated in serum free DMEM for 24 h. Cell death rate was measured using a LDH assay kit. $n = 6$, $^*P < 0.05$ vs. control (−). Right panel: representatives of Nrf2 expression in INS-1 cells transfected with Ctr RNAi and Nrf2 RNAi, which were cultured in serum free RPMI 1640 in the presence of dh404 (300 nM) for 24 h.

of Nrf2 in the diabetic islets. To further verify the findings, we determined mRNA expression of Nrf2 and its downstream target gene NAD(P)H:quinone oxidoreductase (NQO)-1 in the murine normal and diabetic islets by Q-PCR analysis. As shown in Fig. 1B, the expression of Nrf2 and NQO-1 was significantly upregulated. Therefore, these results indicate that Nrf2 is most likely activated in islets at the early stage of diabetes.

3.2. Dh404 activates Nrf2 in β-cells and protects against hydrogen peroxide (H_2O_2)-induced β-cell death and islet damage via activating Nrf2

To investigate the pathophysiological significance of Nrf2 upregulation in diabetic islets, we examined the impact of Nrf2 activation by dh404 on oxidative stress-induced β-cell death and islet damage, most frequently seen in diabetic setting. Oxidative stress-induced β-cell death and islet injury were established by exogenous administration of H_2O_2 in cultured islets as previously described [22]. As expected, H_2O_2 treatment enhanced low serum-induced injury of islets over time and dh404 pretreatment dramatically attenuated the H_2O_2-induced islet damage (Fig. 2A). In addition, H_2O_2 treatment resulted in a substantial increase in the number of apoptotic cells, which was coincident with the dramatic decrease in the number of cells positively stained with pro-insulin and insulin (Fig. 2B and C), suggesting that H_2O_2 induces β-cell death and/or β-cell dysfunction. Importantly, dh404 pretreatment significantly suppressed H_2O_2-induced apoptosis as well as H_2O_2-decreased the number of pro-insulin and insulin positive cells (Fig. 2B and C). These results demonstrate that dh404 is protective against oxidative stress-induced β-cell death and islet damage in vitro. Moreover, dh404 and H_2O_2 individually as well as additively augmented the protein expression of Nrf2 and its downstream gene NAD(P)H:quinone oxidoreductase (NQO)1 in islets (Fig. 3A and Supplementary Fig. 3), suggesting dh404- and/or H_2O_2-induced islet Nrf2 activation. To support these observations, we determined the effects of dh404 and/or H_2O_2 on cytosolic and nuclear Nrf2 expression in islets. Immunofluorescent staining and Western blot demonstrated that dh404 and/or H_2O_2 upregulated both cytosolic and nuclear Nrf2 expression in islets, predominantly in the cytosol (Fig. 3B). It is worthy to note that the unusual pattern of Nrf2 expression as a transcription factor is mostly likely due to the unique mechanism of dh404-mediated Nrf2 activation [21]. The protein stability and transcriptional activity of Nrf2 is principally regulated by its endogenous inhibitor, Keap1 that binds to Nrf2 thereby facilitating its degradation via proteasomes; however, we have uncovered that dh404 does not dissociate the interaction of Keap1 and Nrf2 but inhibit the ability of Keap1 to target Nrf2 for proteasome-mediated degradation [21]. Thus, newly synthesized Nrf2 proteins saturate the capacity of Keap1 binding with Nrf2, accumulate in the cytosol and subsequently translocate into the nucleus, thereby facilitating Nrf2-mediated protection against oxidative stress [21]. Collectively, these results indicate that Nrf2 may play a critical role in mediating the dh404-induced cytoprotection in β-cells. To test this hypothesis, we applied Nrf2 RNAi approach in INS-1 β-cells. Although H_2O_2 activated Nrf2 in INS-1 β-cells (data not shown), the H_2O_2-induced cell death in the cells was hardly affected by knockdown of Nrf2 (Fig. 3), indicating that Nrf2 activation secondary to oxidative stress may not be potent enough to initiate a negative feedback protection or does not lead to cytoprotection at all in β-cells. Nevertheless, dh404 potently suppressed H_2O_2-induced cell death in INS-1 β-cells transfected with control scramble oligonucleotides, the cytoprotective effect of dh404 almost disappeared in the cells transfected with Nrf2 siRNA oligonucleotides (Fig. 3C), suggesting a critical mediator role of Nrf2 for dh404-induced cytoprotection in β-cells against oxidative stress.

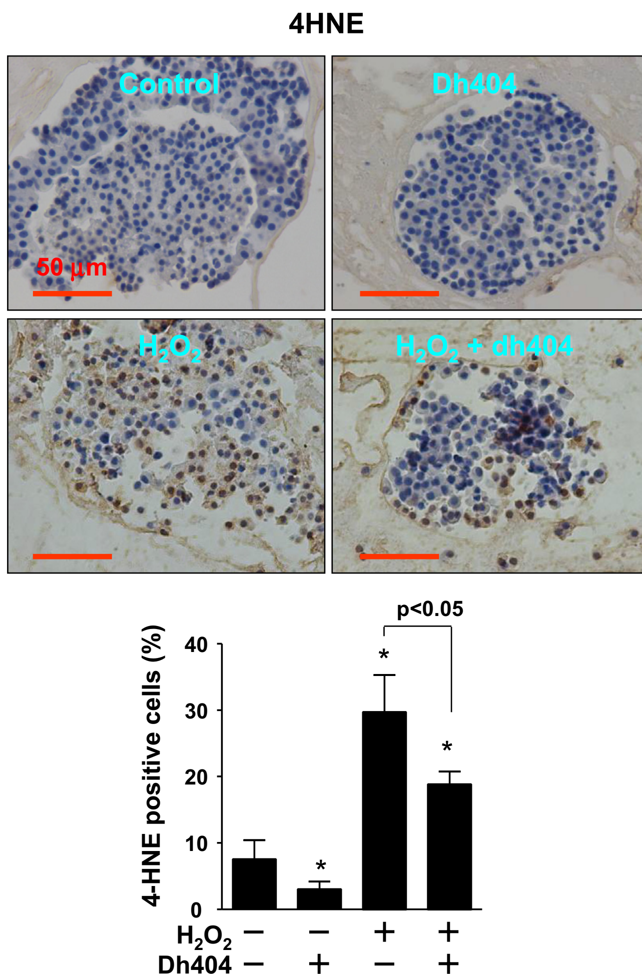

Fig. 4. Effect of dh404 on H_2O_2-induced expression of 4HNE in islets. Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. The islets were then subjected to 4HNE staining. Percentage of positive staining was calculated based on the total number of counter-stained cells ($n = 3$). $^*P < 0.05$ vs. control (−).

3.3. Dh404 suppresses H_2O_2-induced oxidative stress and ubiquitination while enhancing autophagic activity in islets

To explore the molecular mechanism by which dh404 suppresses oxidative stress-mediated β-cell death and islet injury, we first examined whether dh404 pretreatment inhibits H_2O_2-induced oxidative stress in the isolated islets. As expected, dh404 pretreatment suppressed basal and H_2O_2-induced expression of 4HNE, a biomarker of oxidative stress in the islets (Fig. 4). Considering the potent activation of Nrf2 by dh404 treatment in islets (Fig. 3) and a central role of Nrf2 in the control of oxidative stress aforementioned, it is likely that dh404 activates Nrf2 to suppress oxidative stress thereby conferring protection against oxidative stress-induced islet injury.

However, as the magnitude of dh404-induced suppression of 4HNE is relatively less by comparison to the increases in NQO1 expression (Nrf2 activation) (Fig. 3 and Supplementary Fig. 3), it is possible that other Nrf2-operated mechanisms contribute to the dh404-induced protection in islets. Given Nrf2 mediates the autophagic clearance of toxic ubiquitinated proteins secondary to ROS formation in macrophages [16], we postulated that dh404 drives Nrf2 to enhance autophagic clearance of toxic ubiquitinated proteins in islets in addition to suppressing oxidative stress.

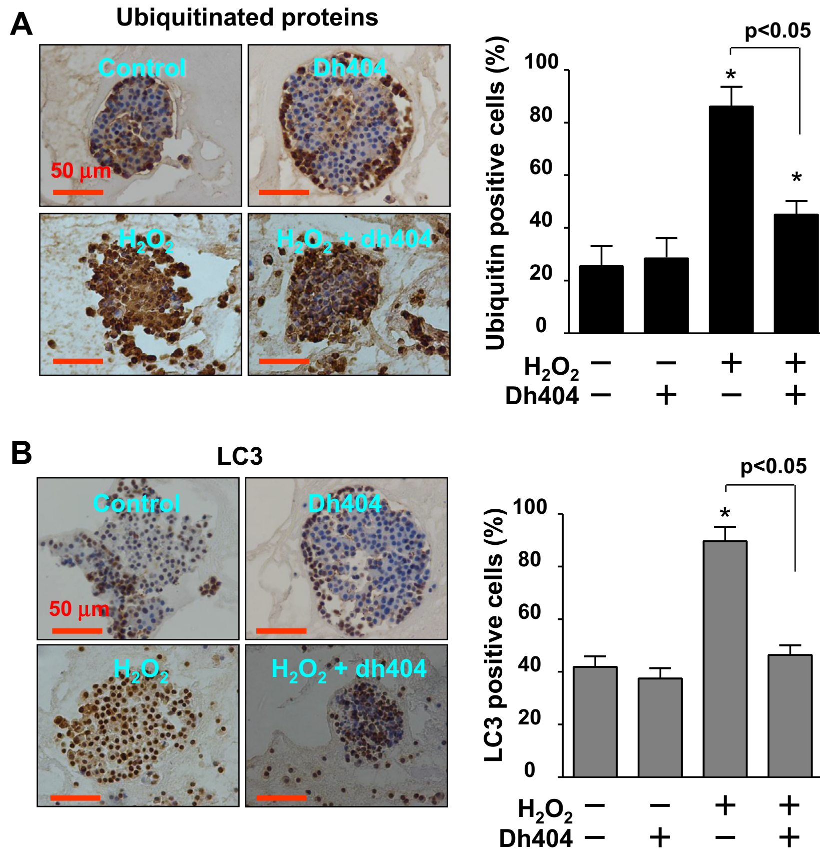

Fig. 5. Effect of dh404 on H_2O_2-induced accumulation of ubiquitinated proteins and expression of LC3 in islets. Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. The islets were then subjected to ubiquitin and LC3 staining. Percentage of positive staining was calculated based on the total number of counter-stained cells (n = 3).*P < 0.05 vs. control (−).

Accordingly, we determined the effect of dh404 on H_2O_2-induced protein ubiquitination and autophagic activity in islets. Immunochemical staining showed that H_2O_2 treatment induced accumulation of ubiquitinated proteins and upregulation of microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagosome [15], in most of the islet cells (Fig. 5), suggesting that H_2O_2-induced oxidative stress causes accumulation of toxic ubiquitinated protein aggregates associated with autophagic activation in β-cells as previously reported [13]. Of interest, dh404 pretreatment suppressed both the accumulation of ubiquitinated proteins and the upregulation of LC3 in H_2O_2-stressed islets (Fig. 5), raising a possibility that dh404 could suppress accumulation of toxic ubiquitinated protein aggregates via enhancing autophagic clearance in islets.

As a result of autophagic activation, native LC3 (LC3-I), a 16-kDa mammalian homologue of yeast autophagy-related gene (Atg) 8, is processed and lipid conjugated resulting in LC3-II, a 14-kDa active isoform that migrates from the cytoplasm to autophagosomes. Thus, LC3-II is considered to be an accurate marker of autophagosome. Because the protein abundance of LC3-II usually reflects the steady level of autophagosomes, which is dependent on a balance between autophagosome synthesis and autophagosome clearance via lysosomes [15], we further determined the effect of dh404 on expression levels of poly-ubiquitinated proteins, LC3-I and II, and p62, a critical adaptor for engaging autophagosome fusion with lysosome thereby leading to autophagic clearance of toxic ubiquitinated protein aggregates [23], in the H_2O_2-stressed islets by Western blot analysis. Consistent with the immunochemical staining findings, we observed that dh404 treatment suppressed H_2O_2-induced accumulation of ubiquitinated proteins while downregulating the expression levels of LC3-II and p62 (Fig. 6), supporting that dh404 is capable of enhancing autophagic clearance of ubiquitinated toxic protein aggregates in islets.

3.4. An essential role of Nrf2 in dh404-induced autophagic clearance of ubiquitinated toxic protein aggregates in β-cells

To determine whether dh404 suppresses the accumulation of ubiquitinated toxic protein aggregates via enhancing Nrf2-mediated autophagic clearance, we examined the impact of Nrf2 knockdown on protein aggregate accumulation and autophagic activity in H_2O_2-stressed INS-1 β-cells treated with or without dh404. It has been well established that autophagy and ubiquitin proteasome system (UPS) are the major routes for the complete degradation/clearance of abnormal protein products in cells [24,25]. UPS is usually effective in clearing soluble misfolded or damaged proteins via ubiquitination of the target proteins whereas autophagy is generally efficient in clearing less soluble or insoluble toxic ubiquitinated protein aggregates. Thus, we first measured the effect of H_2O_2 and dh404 on the expression levels of poly-ubiquitinated proteins, LC3, and p62 in detergent soluble and insoluble fractions of INS-1 cells. Serum starvation time-dependently induced accumulation of ubiquitinated proteins in insoluble fraction, associated with increased levels of LC3-II and p62 in both soluble and insoluble fractions (Supplementary Fig. 4A), suggesting that serum starvation activates autophagic clearance of toxic ubiquitinated protein aggregates in β-cell over time. The serum starvation-induced accumulation of ubiquitinated protein aggregates as well as upregulation of LC3-II and p62 were enhanced by H_2O_2 treatment; however, the magnitude of LC3-II and p62 upregulation by

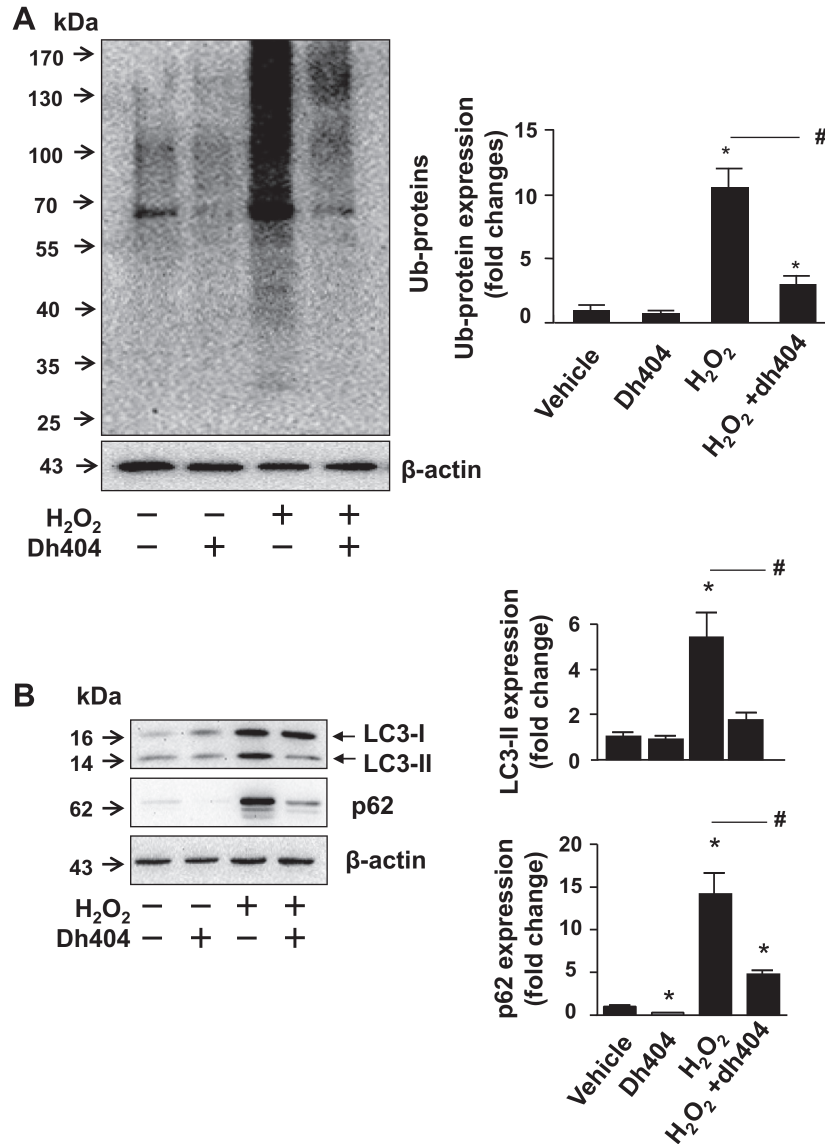

Fig. 6. Effect of dh404 on H_2O_2-induced accumulation of ubiquitinated proteins as well as expression of LC3 and p62 in islets. Isolated rat islets were treated with or without H_2O_2 (150 μM) and dh404 (300 nM) as indicated for 24 h. The islets were then subjected to Western blot analysis of (A) poly-ubiquitinated proteins (Ub-proteins) and (B) LC3-I & -II and p62. Left panel: representatives of immunoblots. Right panel: bar graphs of semi-quantified densitometric analysis. $n = 3$, $^*P < 0.05$ vs. control (−). $^{\#}P < 0.05$.

H_2O_2 was less in insoluble fraction compared with soluble fraction (Supplementary Fig. 4A). These results suggest that H_2O_2 enhances cellular utilization of LC3-II and p62, reflecting increased autophagic clearance of toxic ubiquitinated protein aggregates in β-cells. Moreover, given the time-dependently cell death in H_2O_2-stressed INS-1 cells, it is likely that the H_2O_2-increased autophagic clearance is not sufficient enough to protect the cells against ubiquitinated protein aggregate-induced cytotoxicity. On the other hand, dh404 treatment for 24 h dramatically suppressed the accumulation of ubiquitinated proteins and upregulation of LC3-II and p62 in both soluble and insoluble fractions induced by serum starvation (Supplementary Fig. 4B), suggesting that dh404 could facilitate autophagic clearance of toxic ubiquitinated protein aggregates in β-cells. Utilizing the optimized experimental conditions, we then determined the effect of dh404 on H_2O_2-induced accumulation of ubiquitinated toxic protein aggregates as well as the expression of levels of LC3-II and p62 in insoluble fraction of INS-1 cells transfected with control scramble or Nrf2 siRNA oligonucleotides. As shown in Fig. 7, the inhibitory effect of dh404 on H_2O_2-induced accumulation of ubiquitinated protein aggregates as well as upregulation of LC3-II and p62 were blocked by Nrf2 knockdown, demonstrating an essential role of Nrf2 for dh404-induced suppression of ubiquitinated protein aggregate accumulation and enhancement of autophagic clearance in β-cells.

To further establish a direct link between Nrf2 and dh404-induced autophagic clearance of ubiquitinated protein aggregates in β-cells, we applied chloroquine (CQ), an inhibitor of autophagosome fusion with lysosome. In control cells, CQ treatment resulted in dramatically accumulation of ubiquitinated protein aggregates in insoluble fraction as well as LC3-II and p62 in both soluble and insoluble fractions in the presence of H_2O_2 plus dh404 (Fig. 8). However, the CQ-induced effects were significantly suppressed in Nrf2 knockdown cells (Fig. 8). These results demonstrate that Nrf2 is an essential mediator for dh404-induced autophagic clearance of ubiquitinated protein aggregates in β-cells.

Taken together, these results suggest that dh404 facilitates autophagic clearance of toxic ubiquitinated protein aggregates via Nrf2 activation thereby preventing oxidative stress-mediated

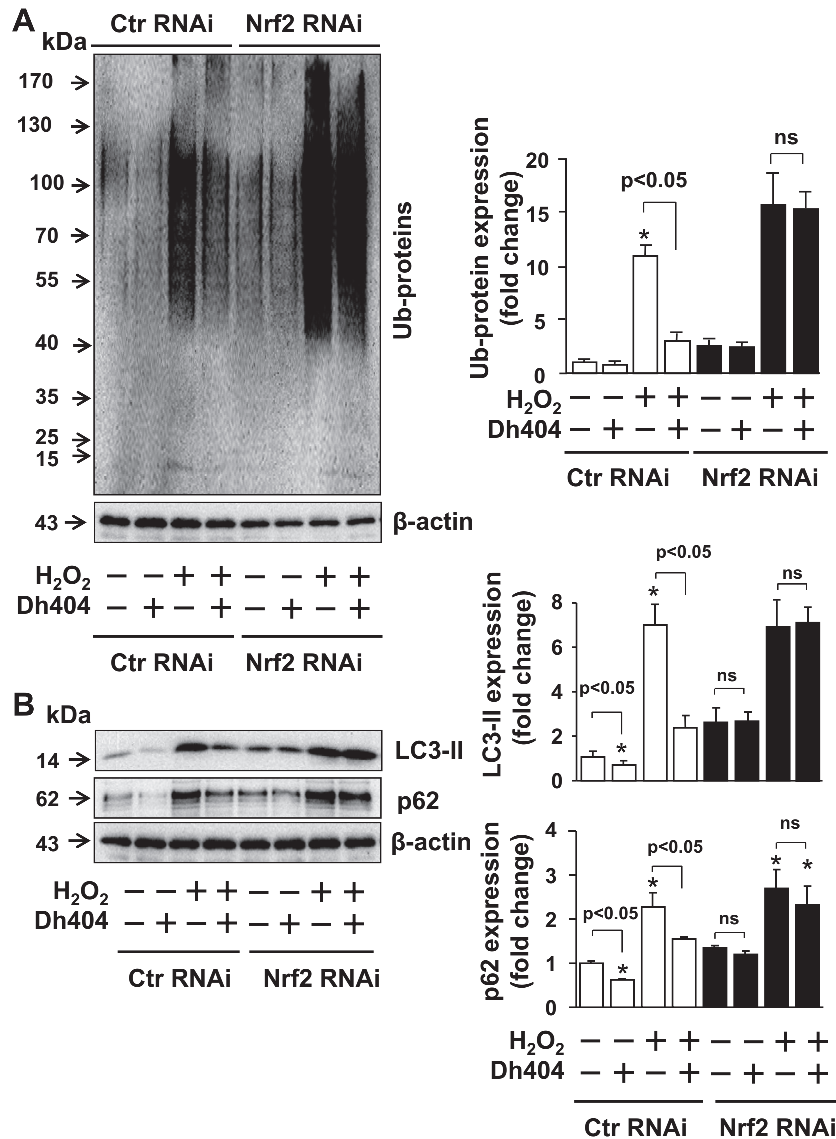

Fig. 7. Impact of Nrf2 knockdown on dh404-suppressed accumulation of ubiquitinated protein aggregates and dh404-induced utilization of LC3 and p62 in INS-1 β-cells in a setting of oxidative stress. INS-1 cells transfected with control scramble oligonucleotides (Ctr RNAi) and Nrf2 RNAi oligonucleotides (Nrf2 RNAi) were treated with or without H_2O_2 (100 μM) and/or dh404 (300 nM) as indicated in serum free RPMI 1640 for 24 h. Detergent insoluble fractions of the cell lysates were subjected to Western blot analysis of poly-ubiquitinated proteins (Ub-proteins), LC3-II, and p62. The efficacy of Nrf2 RNAi was confirmed by Western blot analysis of Nrf2 and NQO-1 (data not shown). (A and B) Left panels: representatives of immunoblots. Right panels: bar graphs of semi-quantified densitometric analysis. $n = 4$, $^*P < 0.05$ vs. control (–).

β-cell damage. Given that the activation of autophagy is secondary to oxidative stress and dh404 activates Nrf2 to suppress oxidative stress while enhancing autophagic clearance of ubiquitinated proteins in islets, it is conceivable that dh404 facilitates the autophagic clearance via driving an antioxidant independent activation of Nrf2 as aforementioned.

4. Discussion

In this study, we have demonstrated for the first time that Nrf2 expression is upregulated in islets at an early stage of diabetes in both mice and humans, thus indicating the clinical protective relevance of Nrf2 in the pathogenesis of human diabetes. Pharmacological activation of Nrf2 by dh404 leads to suppression of β-cell death and islet injury in a setting of oxidative stress. Mechanistically, our findings reveal that dh404 not only activates Nrf2-mediated suppression of oxidative stress but also facilitates an antioxidant independent Nrf2-operated autophagic clearance of ubiquitinated proteins in islets.

Considering the key role of Nrf2 in antioxidant defense [17], it was not surprising to find that the Nrf2 activator dh404 suppresses H_2O_2-induced oxidative stress and accumulation of ubiquitinated proteins in islets, β-cell death, and islet injury. Just as administration of CDDO-Im, an analog of dh404, protected β-cells function presumably via enhancing Nrf2-mediated antioxidant defense in type II diabetic mice [7], we further provided direct evidence that pharmacological activation of Nrf2 by dh404 prevents oxidative stress-mediated β-cell death. However, the enhancement of autophagic clearance of ubiquitinated proteins in the H_2O_2-stressed islets by dh404 is intriguing. In general, reactive oxygen species (ROS) is a mediator of the induction of autophagy and the overproduced ROS could activate autophagy to serve as a predominantly prosurvival mechanism in a setting of oxidative stress [26,27]. In fact, it has been demonstrated that autophagy is activated and

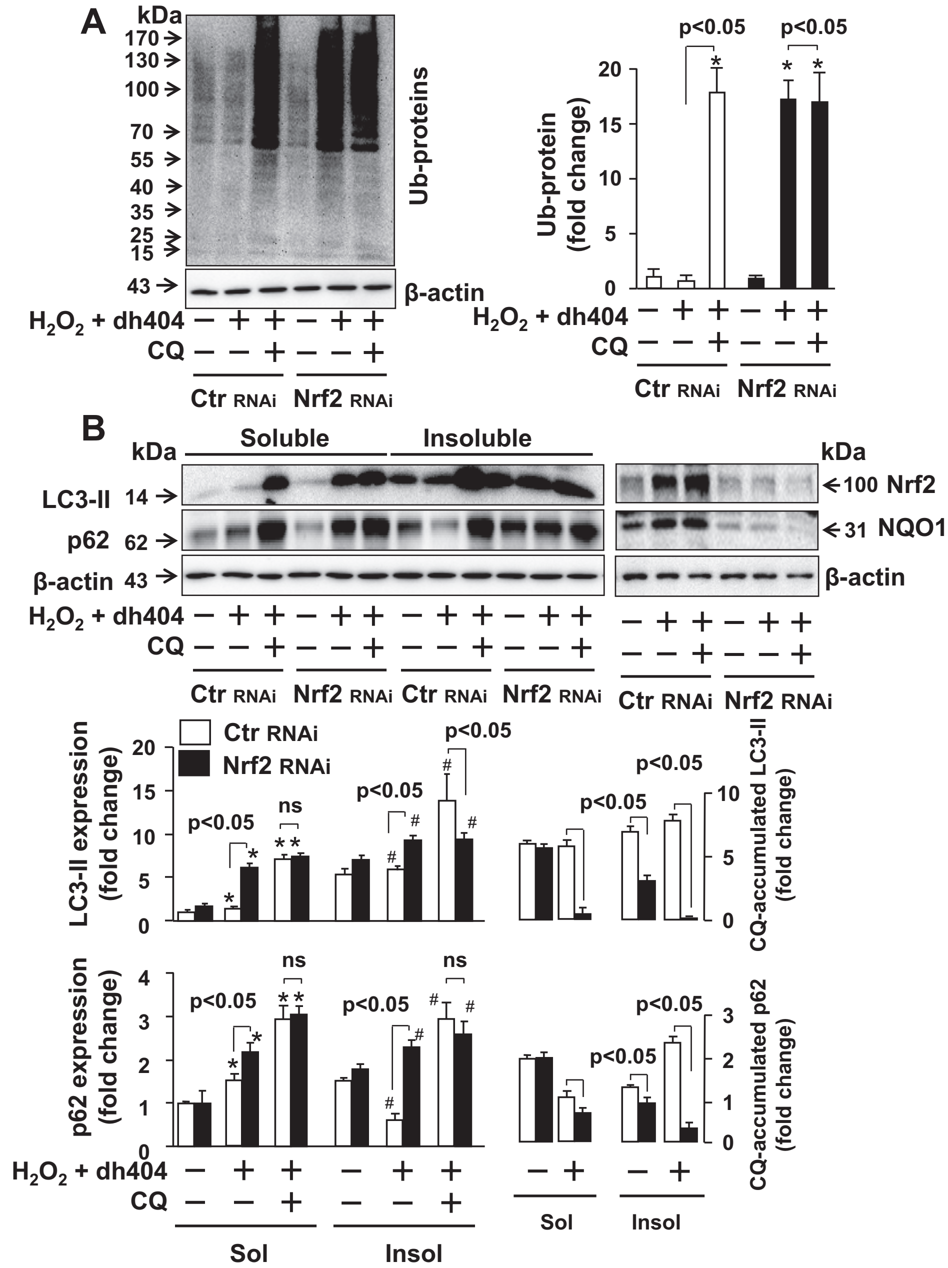

Fig. 8. Impact of Nrf2 knockdown on chloroquine (CQ)-accumulated ubiquitinated protein aggregates, LC3, and p62 in INS-1 cells in the presence of H_2O_2 plus dh404. INS-1 cells transfected with control scramble oligonucleotides (Ctr RNAi) and Nrf2 RNAi oligonucleotides (Nrf2 RNAi) were treated with or without H_2O_2 (100 μM) plus dh404 (300 nM) as indicated in serum free RPMI 1640 for 24 h. CQ (10 μM) was added during the last 6 h. (A) Detergent insoluble fractions of the cell lysates were subjected to Western blot analysis of poly-ubiquitinated proteins (Ub-proteins). Left panel: representative of immunoblots. Right panel: bar graphs of semi-quantified densitomertic analysis. $n = 4$, $^*P < 0.05$ vs. control (−). (B) Detergent soluble and insoluble fractions were subjected to Western blot analysis of LC3-II and p62. The efficacy of Nrf2 RNAi was confirmed by routine Western blot analysis of Nrf2 and NQO-1. Densitometric analysis was shown in Supplementary Fig. 6. Upper panels: representatives of immunoblots. Lower panels: bar graphs of semi-quantified densitometric analysis. $n = 4$, $^*P < 0.05$ vs. control (−).

serves as a negative feedback mechanism to clear toxic ubiquitinated protein aggregates in β-cells in diabetic settings including oxidative stress [13,14]. Therefore, it is presumable that suppression of ROS formation or oxidative stress should inhibit autophagy activation. However, we observed that activation of Nrf2 by dh404 suppressed oxidative stress while enhancing the autophagic activity in islets, suggesting that dh404 drives Nrf2 to activate autophagy via a mechanism independent of its antioxidant effects.

Clinically, this dh404-operated Nrf2-mediated antioxidant independent activation of autophagy in islets is particularly interesting. As the islet might be the most vulnerable tissue to oxidative stress [3,4], forced activation of Nrf2 in the islet may not only suppress the source of pancreatic oxidative stress but also repair the established oxidative stress-induced islet damage thereby restoring islet function. Given dh404 is well tolerated in rodents and primates and suppresses the onset of several diseases in animal models [28], it is positioned to be a promising drug target for maximal activation of Nrf2-driven cytoprotective signaling in islets.

Recent studies have revealed that Nrf2 cross-talks with other molecular pathways; consequently, the role of Nrf2 in biological systems/pathological conditions may have other protective abilities besides its antioxidant effects [5]. Several dh404 analogs have indicated pleiotropic effects independent of Nrf2 signaling [29–31].

Since the molecular mechanism of Nrf2-mediated autophagic clearance of toxic ubiquitinated proteins in β-cells remains unknown, the precise contribution of Nrf2 and other potential signaling in dh404-induced protection of β-cell function need to be further investigated. The outcome will provide valuable information to validate Nrf2 as a key target for prevention and attenuation of diabetes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.04.046.

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