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Research Paper

Mild exposure of RIN-5F β -cells to human islet amyloid polypeptide aggregates upregulates antioxidant enzymes via NADPH oxidase–RAGE: An hormetic stimulus[☆]



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ABSTRACT

The presence of amyloid aggregates of human islet amyloid polypeptide (hIAPP), a hallmark of type 2 diabetes, contributes to pancreatic β -cell impairment, where oxidative stress plays a key role. A contribution of NADPH oxidase to reactive oxygen species (ROS) generation after cell exposure to micromolar concentrations of hIAPP aggregates has been suggested. However, little is known about β -cells exposure to lower amounts of hIAPP aggregates, similar to those found in human pancreas. Thus, we aimed to investigate the events resulting from RIN-5F cells exposure to nanomolar concentrations of toxic hIAPP aggregates. We found an early and transient rise of NADPH oxidase activity resulting from increased Nox1 expression following the engagement of receptor for advanced glycation end-products (RAGE) by hIAPP aggregates. Unexpectedly, NADPH oxidase activation was not accompanied by a significant ROS increase and the lipoperoxidation level was significantly reduced. Indeed, cell exposure to hIAPP aggregates affected the antioxidant defences, inducing a significant increase of the expression and activity of catalase and glutathione peroxidase. We conclude that exposure of pancreatic β -cells to nanomolar concentrations of hIAPP aggregates for a short time induces an hormetic response via the RAGE–Nox1 axis; the latter stimulates the enzymatic antioxidant defences that preserve the cells against oxidative stress damage.

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Introduction

hIAPP is a peptide hormone co-secreted with insulin by pancreatic β -cells with a glucomodulatory role in animal metabolism [1]. The presence of fibrillar deposits of hIAPP amyloid aggregates in the islets of Langerhans is a recognised hallmark of type 2 diabetes which is strongly related to pancreatic β -cell suffering and death, a fundamental feature of the disease [2,3].

Abbreviations: hIAPP, human islet amyloid polypeptide; rIAPP, rat islet amyloid polypeptide; ROS, reactive oxygen species; RAGE, receptor for advanced glycation end-products; AGE, advanced glycation end products; DPI, diphenyleneiodonium; MDA, malonyldialdehyde; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ATZ, 3-amino-1,2,4-triazole; MS, mercaptosuccinic acid

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hIAPP fibrillar aggregates result from peptide misfolding, possibly favoured by the presence of lipid membranes or other biological surfaces, with structural reorganisation into early unstable oligomeric assemblies displaying a strong tendency to interact with the cell surface [4,5]. Amyloid oligomers further grow into increasingly ordered polymers eventually generating straight, unbranched, 6–10 nm wide, several μ m long, β -structure enriched fibrils [6].

Growing evidence suggests that oxidative stress plays a key role in the onset of type 2 diabetes [7]. In addition, β -cells express low physiological levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [8,9]. Many sources of ROS contributing to the development of type 2 diabetes have been described: a high level of mitochondrial oxidative phosphorylation, glucose auto-oxidation, production of Advanced Glycation End products (AGEs), overexposure to Angiotensin II (which increases NADPH oxidase activity) [10,11] and cell exposure to hIAPP aggregates which induce some alteration of membrane permeability [10,12]. The importance of ROS increase as a cause of cell suffering during exposure to high concentrations of hIAPP aggregates has been indirectly confirmed by the

protection provided by antioxidants; the latter were found to improve the viability of either RINm5F β -cells and INS-1E β -cells exposed to 10 μ M hIAPP aggregates [13] and to 10–30 μ M hIAPP aggregates [14], respectively.

A problem with *in vitro* studies is that usually hIAPP doses are far from those normally found in the human pancreas; in fact, hIAPP can reach a millimolar concentration inside intracellular storage granules [15] but it is rapidly diluted outside the cell reaching picomolar concentrations in the plasma [16]. So, even though a local increase of hIAPP concentration in the islets can be postulated in the context of amyloid aggregation, it is hard to accept that it could reach micromolar levels. Then, we aimed to investigate the molecular and cellular events associated with the exposure of RIN-5F β -cells to nanomolar concentrations of hIAPP aggregates. In particular, we were interested in assessing the contribution of NADPH oxidase as a source of ROS at these experimental conditions, since its involvement in oxidative stress in cells exposed to 10–30 μ M hIAPP had already been suggested [12].

NADPH oxidase is an ubiquitous multi-subunit enzyme which generates superoxide from molecular oxygen using NADPH as the electron donor. NADPH oxidase was originally identified in phagocytes, where it consists of two membrane-associated subunits, p22^{phox} and gp91^{phox} (renamed Nox2), and at least four cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox} and Rac1/2), which translocate to the membrane upon enzyme activation. Seven isoforms of the catalytic subunit Nox (Nox1-5, Duox 1 and 2), with different cellular localisation, tissue distribution and expression are known [17–19]. The RINm5F pancreatic β -cells (of which RIN-5F is a subclone) express only Nox1 and Nox4 [20]. Nox4 is constitutively expressed and active, whereas Nox1, like the Nox2 isoform, requires other components for activity. In fact, Nox1 is constitutively associated with NoxO1 and NoxA1, the p47^{phox} and p67^{phox} homologues, respectively [19].

In this study we report the effect of cell exposure to low concentrations of hIAPP aggregates for short time periods. Our results led us to uncover an unexpected hormetic effect of such treatment that involved NADPH oxidase activation via RAGE yet resulting in cell protection against oxidative stress through a significant increase of the enzymatic antioxidant defences.

Material and methods

Cell culture

Rat RIN-5F insulinoma cells, from American Type Culture Collection (ATCC), were cultured as described previously [21].

Production of hIAPP amyloid aggregates

hIAPP (Calbiochem, La Jolla, CA) was dissolved in 80% hexafluoroisopropanol (HFIP, Sigma-Aldrich, Steinheim, Germany) to a concentration of 512 μ M and stored at -20°C until use. hIAPP aggregates were prepared by diluting hIAPP to 6.5 μ M in 10 mM phosphate buffer, pH 7.4, 1.0% HFIP, and by incubating the mixture at 25°C for 30 min [21]. Aggregate morphology was determined by Transmission Electron Microscopy (TEM) as previously described [21]. rIAPP (Bachem, Bubendorf, Switzerland) was dissolved and incubated at the same conditions as hIAPP.

Cell treatments

Freshly prepared hIAPP aggregates were diluted in cell culture medium without phenol red, containing 0.5% FCS, and administered to RIN-5F cells. Cells were also treated with similarly diluted

rIAPP. In some experiments, the cells were pre-treated for 1 h with (i) the NADPH oxidase inhibitors diphenyleneiodonium (DPI, 10 μ M) and apocynin (Apo, 100 μ M); (ii) a blocking anti-RAGE antibody (sc-5563, Santa Cruz Biotech), 20 μ g/mL; (iii) the CAT and GPx inhibitors 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS) (20 mM and 7.0 mM, respectively) [22]. All inhibitors were from Sigma. Experiments were carried out to optimise inhibitor concentrations.

Cell viability assessment

20,000 cells/well were seeded into 96-well plates in complete medium, cultured for 48 h and then treated for 3 h with hIAPP aggregates diluted to 60 nM (here and thereafter, aggregate concentration refers to the monomeric peptide concentration). Cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described [21]; cell mortality was assessed by determining the Lactate Dehydrogenase (LDH) release in the culture medium with the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Italy) [23].

NADPH oxidase activity determination by lucigenin chemiluminescence

The cells were treated with 60 nM hIAPP aggregates for 0–4.5 h and with 60, 120 or 500 nM hIAPP, or with 120 nM rIAPP for 3.0 h. After washing twice with PBS, the cells were harvested, incubated for 60 min in 20 mM ice-cold Tris-HCl buffer, pH 7.4, containing 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1.0 mM PMSF and phosphatase inhibitors (Sigma, Italy) and sonicated to obtain total cell lysates. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Italy). Superoxide generation was assayed at room temperature using lucigenin (5.0 μ M)-enhanced chemiluminescence, as previously described [24]. Each measurement was performed in 20 mM Tris HCl, pH 7.4 with 30–60 μ g of total cell lysate proteins in the absence or in the presence of specific oxidase substrates (300 μ M NADPH for NADPH oxidase, 5.0 mM succinate for mitochondrial oxidase complex I or 1.0 mM xanthine for xanthine oxidase). Further measurements were performed after the addition of the NADPH oxidase inhibitors DPI (20 μ M) and Apo (100 μ M). A buffer blank was subtracted from each reading. Superoxide production was detected using a Lumat LB 9507 luminometer (EG&G Berthold) and calculated as an arbitrary light unit (MLU) over 10 min.

Intracellular ROS content and lipid peroxidation assay

Intracellular ROS content was measured by monitoring the oxidation of 2',7'-dihydrodichlorofluorescein diacetate (H₂DCF-DA, Invitrogen, Italy) to the fluorescent dichlorofluorescein (DCF), as previously described [25]. The cells were treated with 10 μ M H₂DCF-DA for 10 min before the end of a 3.0 h exposure to 60 nM hIAPP. After washing twice with PBS, the cells were lysed in RIPA buffer (50 mM Tris /HCl buffer, pH 7.5 containing 150 mM NaCl, 2.0 mM EGTA, 100 mM NaF, 2.0% Triton X-100, 1.0 mM vanadate, 0.1% SDS, 2.0 mM EDTA, 2.0 mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin). DCF fluorescence was measured on a Fluoroskan Ascent Fluorometer (Thermo Electron Corporation, Finland), and normalised to the total protein content. Lipid peroxidation was evaluated as malonyldialdehyde (MDA) content using a "Bioxytech LPO-586 Assay" kit (Oxis International Inc., Prodotti Gianni, Italy), according to the manufacturer instructions.

Antioxidant enzyme activities

Total cell lysates were spun at 1500g at 4 °C for 10 min and 70 µg of supernatant proteins were used to determine antioxidant enzyme activities.

CAT activity assay

The samples were diluted in 100 mM phosphate buffer, pH 6.8, containing 10 mM H₂O₂. CAT activity as µmol/min/mg of protein was determined as previously described [26].

GPx activity assay

The samples were diluted in 100 mM phosphate buffer, pH 7.4, containing 0.5 mM EDTA, 1.0 mM NaN₃, 0.25 mM NADPH, 2.25 mM GSH, 1.0 U/ml glutathione reductase. After addition of 0.24 mM *t*-butyl hydroperoxide (Sigma, Italy), the change of the optical density of NADPH was monitored at 340 nm for 2.0 min at 25 °C [27] and GPx activity, as nmol/min/mg of protein, was calculated.

SOD activity assay

SOD activity was evaluated using a Chemical Superoxide Dismutase Assay kit (Cayman Chemical, Ann Arbor, MI, USA) as previously described [27] and calculated as U/mg protein.

Western blotting

The cells were lysed directly in Laemmli buffer (66 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol), boiled for 10 min and clarified at 10,000g for 10 min. Equal protein amounts were separated on 12% SDS-PAGE and transferred to PVDF membranes (Amersham Bioscience, UK). After blocking with 5.0% (w/v) BSA in 0.1% (v/v) PBS-Tween-20, the membranes were incubated overnight at 4 °C with goat anti-Nox1 (1:500, sc-25545), goat anti-Nox4 (1:1000, ab109225), rabbit anti-RAGE (1:500), rabbit anti-CAT (1:1000, sc-50508) and rabbit anti-GPx (1:1000, sc-30147) antibodies. Following 1.0 h incubation with donkey anti-goat (1:10,000, sc-2020) or goat anti-rabbit (1:10,000, sc-2004) secondary antibodies, the immunoreactive bands were detected by the chemiluminescent Immobilon Western HRP Substrate (Millipore, MA, USA) and quantified by densitometric analysis using a ChemiDoc system and the Quantity One software (Bio-RAD Laboratories, Italy). Rabbit anti-GAPDH (1:5000, sc-25778) was used as a reference for equal protein loading. Anti-Nox4 was from Abcam (Cambridge, U.K.), all other antibodies were from Santa Cruz Biotech (Santa Cruz, CA, USA).

Statistical analysis

All data (average of three determinations for each sample) were converted into percentages of the maximum value for each experiment and reported as mean ± SD from at least three independent experiments. Statistical analysis of the data was performed using the Tukey–Kramer test. Differences were considered significant at the $p < 0.05$ level.

Results

NADPH oxidase activity in RIN-5F cells

Many data support the idea that, similarly to amyloid oligomers of other peptides and proteins, hIAPP pre-fibrillar aggregates are also cytotoxic [28,29] and that a derangement of the redox status does occur in exposed cells, although recent evidence suggests that the ability of hIAPP fibrils to distort and disassemble the cell membrane

is also an important feature of oligomer toxicity [30,31]. We used throughout the study early hIAPP aggregates obtained by incubating the peptide for 30 min in aggregation-promoting conditions and whose structure and toxicity were previously characterised extensively by Thioflavine-T assay, CD, FTIR, MTT assay and caspase-3 activity assay [21]. Aggregates were freshly prepared immediately before their administration to cells and TEM analysis confirmed that mature fibrils were not present (Fig. 1). Due to their extreme dilution, it was not possible to perform structural analysis on hIAPP aggregates at the end of the incubation in cell culture, anyway, their interaction with cells in these conditions was previously demonstrated by immunofluorescence analysis [21].

Considering that most studies on hIAPP cytotoxicity were carried out at quite harsh conditions (micromolar concentrations of peptide, long times of cell exposure), the aim of our study was to assess the effect of quite milder, more physiological, conditions on the redox status and viability of cultured RIN-5F pancreatic cells. At first we wanted to determine the contribution of the various enzymatic sources of superoxide in RIN-5F cells. We found a significant superoxide increase in cells, with respect to the basal level measured in the absence of substrates, only in the presence of NADPH (48.04 ± 12.01 MLU/s Vs 16.97 ± 0.66 MLU/s, $p < 0.0001$, $N=10$), whereas superoxide production was negligible in the presence of succinate (16.09 ± 0.67 MLU/s, $N=10$) or xanthine (17.38 ± 3.11 MLU/s, $N=10$), suggesting that NADPH oxidase was likely a major source of superoxide in these cells. Then, we investigated whether NADPH oxidase was involved in the molecular events following cell exposure to nanomolar concentrations of hIAPP aggregates. NADPH -dependent superoxide production was significantly higher in cells exposed for 3.0 h to 60 nM hIAPP aggregates ($p < 0.001$), as compared to cells exposed to vehicle (t_0 value, Fig. 2A). On the contrary, when the cells were exposed for 3.0 h to higher hIAPP concentrations, NADPH oxidase activation was substantially unchanged up to 120 nM hIAPP and declined to the level of vehicle-treated cells at 500 nM hIAPP (Fig. 2B). For this reason, all subsequent investigations were carried out by treating the cells for 3.0 h with 60 nM hIAPP. No significant effect on NADPH oxidase activity was observed in cells exposed for 3.0 h to 120 nM non-amyloidogenic rIAPP, used as a negative control (Fig. 2B). Cell pre-treatment with 10 µM DPI and 100 µM Apo [32–34] before exposure to 60 nM hIAPP aggregates resulted in inhibition of basal and hIAPP-induced NADPH oxidase activity ($p < 0.001$; Fig. 2C) thus confirming enzyme activation.

Nox1 and Nox4 protein expression and role of RAGE

To elucidate the mechanisms underlying the increase of NADPH oxidase activity following cell exposure to hIAPP aggregates, we focused on Nox4 and Nox1, the catalytic subunits expressed in this cell line. No significant change in Nox4 protein expression, as

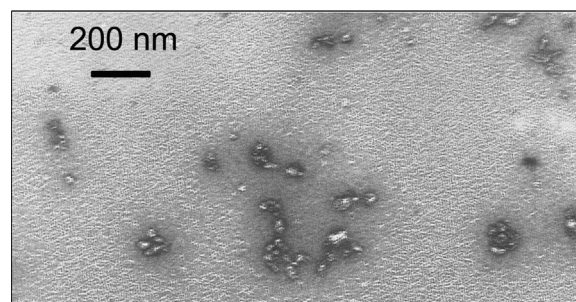


Fig. 1. Characterisation of hIAPP aggregates. TEM imaging of hIAPP aggregates obtained after 30 min of incubation at 6.5 µM under aggregation conditions.

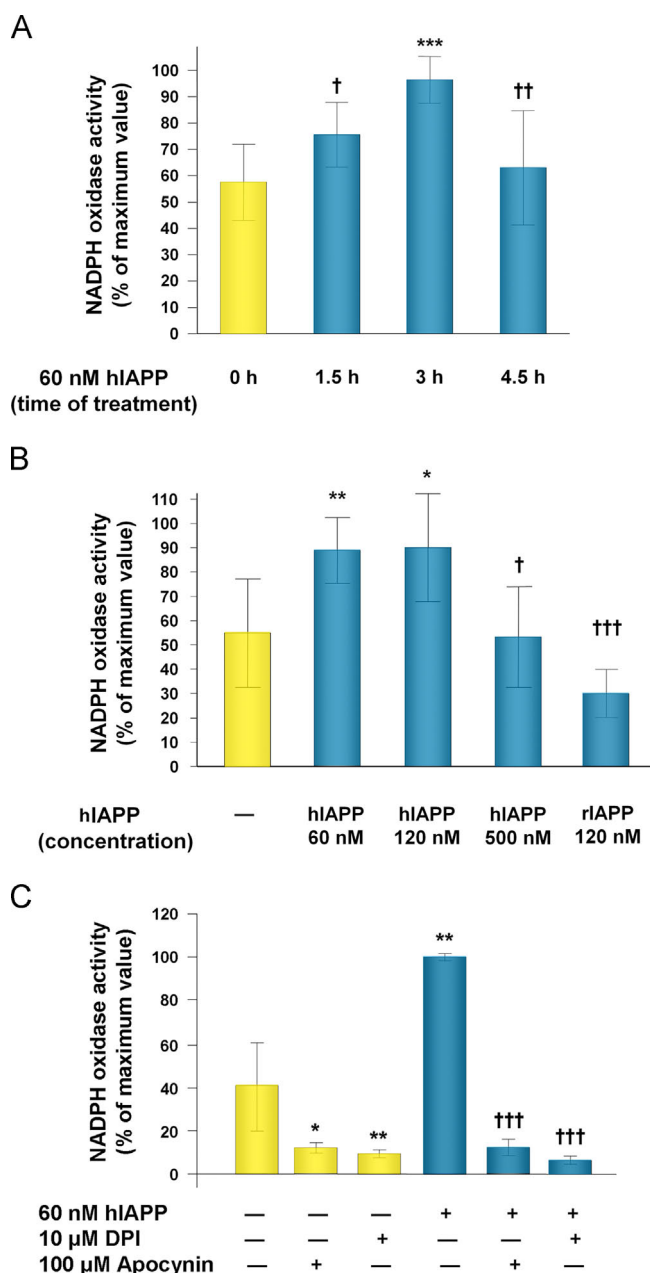


Fig. 2. NADPH oxidase activity increases in RIN-5F cells exposed to hIAPP aggregates. NADPH oxidase activity in RIN-5F cells exposed to 60 nM hIAPP for 0.0, 1.5, 3.0, and 4.5 h (A); NADPH oxidase activity in RIN-5F cells exposed to 60, 120, and 500 nM hIAPP, to 120 nM rIAPP or to vehicle for 3 h (B); NADPH oxidase activity in RIN-5F cells pre-treated or not with 10 μ M DPI or 100 μ M Apo and then exposed to 60 nM hIAPP or to vehicle for 3 h (C). Data are expressed as percentage of maximum value for each experiment and presented as mean \pm SD. * p < 0.05, ** p < 0.01, and *** p < 0.001 Vs vehicle-treated cells; † p < 0.05, †† p < 0.01, and ††† p < 0.001 Vs hIAPP-treated cells. $N=8$, separate experiments. hIAPP, human islet amyloid polypeptide, rIAPP, non-amyloidogenic rodent IAPP. Yellow bars: vehicle-treated cells; blue bars: hIAPP-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assessed by western blot, was observed in hIAPP-treated cells compared to vehicle-exposed cells (Fig. 3B). On the contrary, the level of Nox1 expression was significantly increased in hIAPP-treated cells (p < 0.01; Fig. 3A), suggesting that the increase of NADPH oxidase activity is likely associated to that of Nox1 expression. It has previously been reported that amyloid aggregates bind RAGE [35–37] and that receptor engagement by itself

can generate ROS in part via NADPH oxidase [38]. Therefore, we checked the expression level of RAGE in hIAPP exposed cells and found it was significantly increased with respect to vehicle exposed cells (p < 0.01; Fig. 3C).

To assess whether the previously reported hIAPP-RAGE interaction [36,37] was actually involved in the observed increase of both Nox1 expression and NADPH oxidase activity, we measured such parameters in RIN-5F cells pre-treated with a blocking anti-RAGE antibody before exposure to 60 nM hIAPP aggregates for 3.0 h. These cells displayed a significant decrease both in Nox1 expression (Fig. 4A) and in NADPH oxidase activity (Fig. 4B) (p < 0.01 Vs hIAPP-treated cells). No significant change in both Nox1 protein expression and NADPH oxidase activity was observed when RIN-5F cells were treated with pre-immune non-specific IgG before exposure to 60 nM hIAPP aggregates for 3.0 h (data not shown). These data confirm the RAGE-hIAPP oligomer interaction and suggest that it upregulates the expression of the receptor itself and of Nox1 which, in turn, is responsible for the increase of NADPH oxidase activity.

Enzymatic antioxidant defences and oxidative stress damage

In spite of the observed activation of NADPH oxidase, we did not find a significant decrease of viability (assessed by MTT reduction and LDH release assays) of the cells exposed for 3.0 h to 60 nM hIAPP aggregates compared to vehicle-treated cells (data not shown). These findings led us to hypothesise the occurrence of a buffering effect provided by an increase of the antioxidant defences. Therefore, we measured the activities of CAT and GPx, which catalyse H_2O_2 decomposition, after 3.0 h of cell exposure to hIAPP aggregates. Remarkably, CAT and GPx activities were significantly higher in hIAPP-treated cells with respect to vehicle-exposed cells (p < 0.01 CAT Vs vehicle-treated cells; p < 0.05 GPx Vs vehicle-treated cells). Such an increase was completely abolished when the cells were pre-incubated with DPI or with an anti-RAGE antibody (Fig. 5A and C), while no effect was found in cells not exposed to hIAPP. Moreover, an increase of the expression levels of the two antioxidant enzymes was evident (Fig. 5B and D) that was not present when the cells were pre-treated with DPI. These data suggest a close relationship among the activities of the two antioxidant enzymes and of NADPH oxidase via the hIAPP-RAGE interaction supporting that, at our conditions, transcriptional upregulation of CAT and GPx may be associated with the activation of NADPH oxidase.

At variance with CAT and GPx, SOD activity was not significantly modified at the same experimental conditions (hIAPP-treated cells: 7.18 U/mg \pm 1.07; vehicle-treated cells: 7.17 U/mg \pm 1.23, $N=3$).

This scenario agrees with the results of the analysis of ROS levels in cells exposed for 3.0 h to 60 nM hIAPP: in spite of NADPH oxidase activation, we did not find any significant increase of ROS content in these cells, as compared to vehicle-treated cells (Fig. 6A). To confirm that in hIAPP-exposed cells the reduced oxidative stress in the presence of increased NADPH oxidase activity did result from the activation of the two antioxidant enzymes, we measured ROS levels in the same cells pre-treated for 1.0 h with the CAT inhibitor ATZ (20 mM) or with the GPx inhibitor MS (7.0 mM) and we found a significant increase of ROS content (Fig. 6A), in agreement with previous data [12,14]. When we assayed the level of oxidative stress in terms of lipoperoxidation in hIAPP-exposed cells, we observed a significant decrease of MDA content (Fig. 6B); on the other side, the level of MDA in cells pre-incubated for 1.0 h with a mixture of 20 mM ATZ and 7.0 mM MS before exposure to hIAPP aggregates was not significantly different from that of vehicle-treated cells.

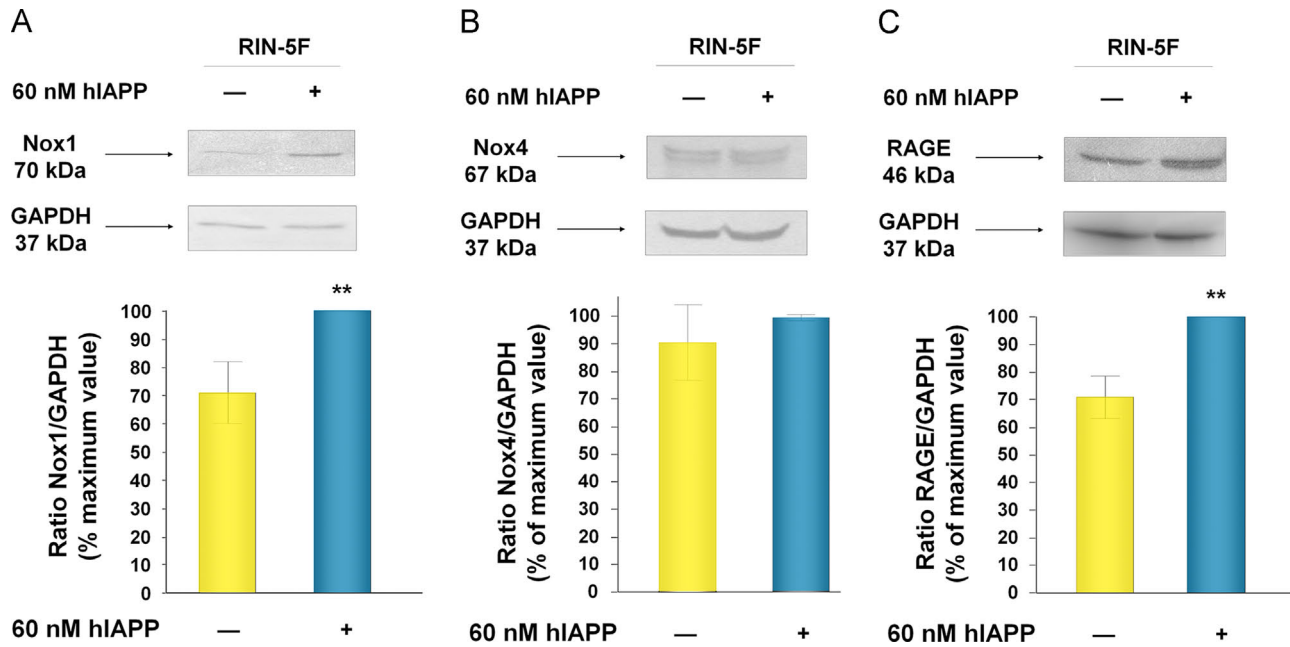


Fig. 3. Nox1, Nox4 and RAGE expression after cell exposure to hiAPP aggregates. Nox1 (A), Nox4 (B) and RAGE (C) expression in RIN-5F cells exposed for 3 h to 60 nM hiAPP or to vehicle. (Upper) Representative immunoblots out of three that gave qualitatively identical results. GAPDH was used to confirm equal protein loading. (Lower) Densitometric quantifications of the Nox1, Nox4 or RAGE ratio to GAPDH protein expression. Data are expressed as percentage of maximum value for each experiment and presented as mean \pm SD. ** $p < 0.01$ Vs vehicle-treated cells. $N=3$, separate experiments. Yellow bars: vehicle-treated cells; blue bars: hiAPP-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

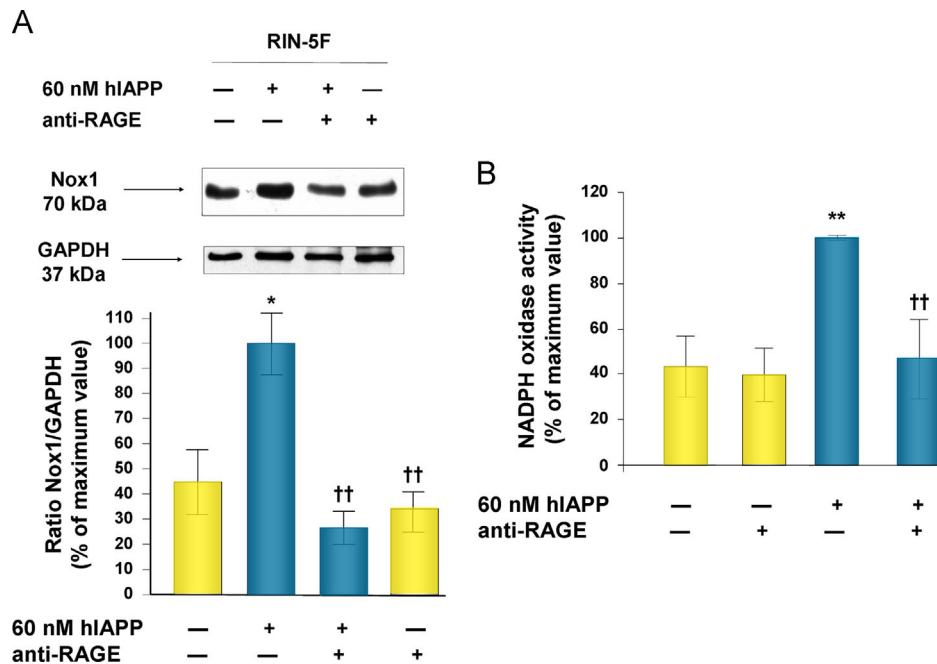


Fig. 4. Effect of cell pre-incubation with anti-RAGE antibody on Nox1 protein expression and NADPH oxidase activity. Nox1 protein expression (A) and NADPH oxidase activity (B) in RIN-5F cells pre-incubated or not with a specific anti-RAGE antibody and then exposed for 3 h to 60 nM hiAPP or to vehicle. (A): (Upper) Representative immunoblot out of five that gave qualitatively identical results; GAPDH was used to confirm equal protein loading; (Lower) densitometric quantification of the Nox1 ratio to GAPDH protein expression. Data are expressed as percentage of maximum value for each experiment and presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ Vs vehicle-treated cells; †† $p < 0.01$ Vs hiAPP-treated cells. $N=5$, separate experiments. Yellow bars: vehicle-treated cells; blue bars: hiAPP-exposed cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Taken together, these data suggest that the amount of ROS produced by NADPH oxidase at these experimental conditions is not harmful to cells but, rather, appears a protective response that serves a signalling function resulting in increased anti-oxidant power.

Discussion

The importance of hiAPP aggregation in type 2 diabetes pathogenesis and the contribution of oxidative stress to hiAPP toxicity, which plays a pivotal role in disease-associated cell dysfunction

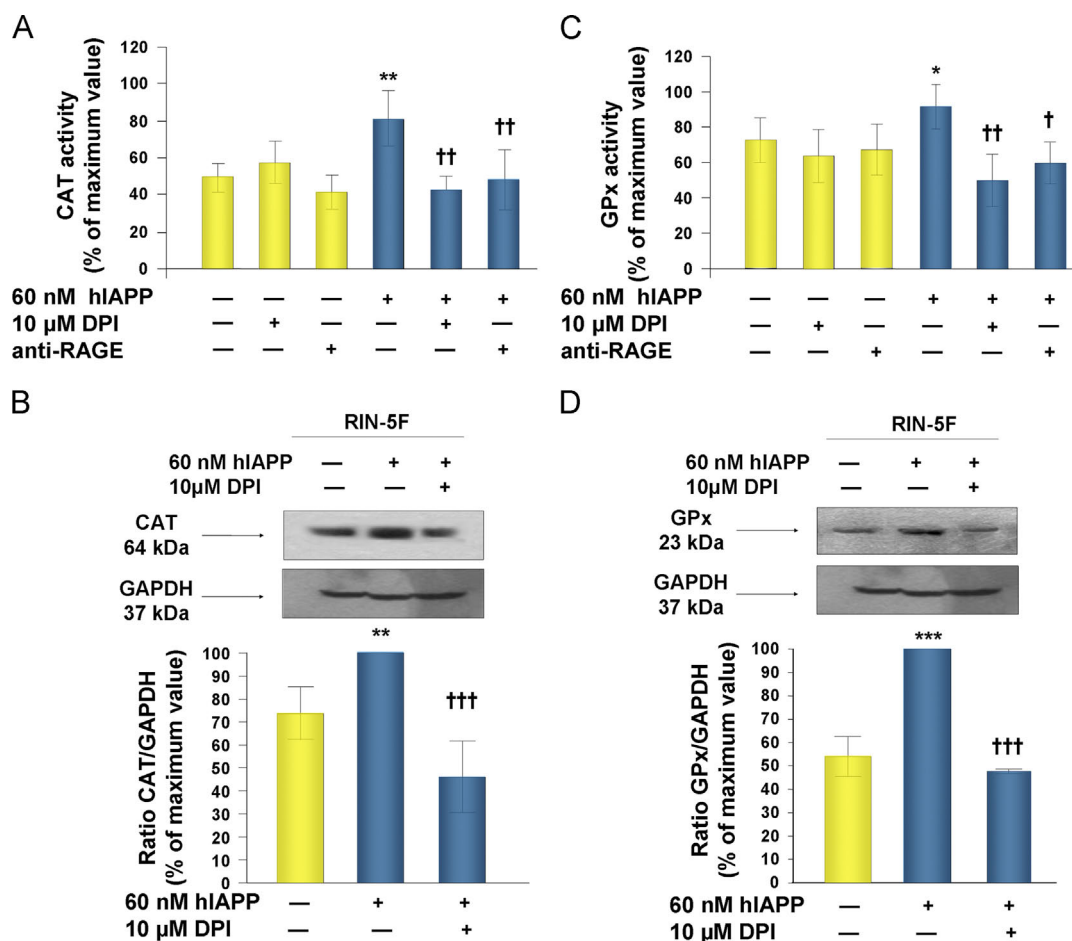


Fig. 5. Enzymatic antioxidant defences in RIN-5F cells exposed to hIAPP aggregates. CAT (A) and GPx (C) enzymatic activities in RIN-5F cells pre-incubated or not in the presence of 10 μM DPI or of a specific anti-RAGE antibody before a 3 h exposure to 60 nM hIAPP aggregates or to vehicle. CAT (B) and GPx (D) protein expression in RIN-5F cells pre-incubated or not with 10 μM DPI before exposure for 3 h to 60 nM hIAPP or to vehicle; (upper) representative immunoblots out of three that gave qualitatively identical results, GAPDH was used to confirm equal protein loading; (lower) densitometric quantification of the CAT or GPx ratio to GAPDH protein expression. Data are expressed as percentage of maximum value and presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Vs vehicle-treated cells; † $p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$ Vs hIAPP-exposed cells. $N = 7$ for (A) and (C); $N = 3$ for (B) and (D), separate experiments. Yellow bars, vehicle-treated cells; blue bars, hIAPP-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and death, is generally accepted [10,12,39], although the details of the phenomenon are still far from being defined. Previous approaches to this problem used hIAPP concentrations (usually in the 10–30 μM range), much higher than those experienced physiologically by the cells in the pancreatic islets [12–14], so that the resulting strong toxic insult could mask the subtle events resulting from the presence of tiny amounts of amyloid aggregates.

In this study we hypothesised that cell treatment with low concentrations of hIAPP aggregates for a short time period recalls more closely the conditions present in the pancreas, particularly in a pre-diabetic phase and at the onset of the pathology. Here we show for the first time: (i) that exposure of pancreatic β-cells to a low concentration of hIAPP amyloid aggregates for a short time increases NADPH oxidase expression and activity; (ii) that such activation depends on RAGE engagement by the aggregates; and (iii) that RAGE-dependent NADPH oxidase activation triggers a protective response through an increase of CAT and GPx expression and activity which counteracts ROS increase. We moved from previous researches showing that different isoforms of NADPH oxidase are expressed in rat islets and in pancreatic β-cells [20,40,41], that they are increased in type 2 diabetes [11,40] and seem to be involved in ROS generation and oxidative stress associated to the pathology [12]. The involvement of NADPH oxidase in the context of various stimuli eliciting oxidative stress in pancreatic cells was indirectly suggested by protection provided

by the antioxidant Apo and by the flavoprotein inhibitor DPI, two commonly used, yet not specific, NADPH oxidase inhibitors [32–34].

Using 60 nM hIAPP aggregates we observed a transitory significant increase in NADPH oxidase activity (by ~50%) after 3.0 h of cell exposure; this was associated to the increase of Nox1 expression (by ~44%), in agreement with previous findings showing that Nox1 is subjected to transcriptional regulation under a variety of stimuli [42]. At variance of Nox1, Nox4 expression was unchanged. Then, we investigated whether the increase of Nox1 expression induced by hIAPP aggregates was mediated by some membrane component. It is known that amyloid aggregates, besides interacting directly and non-specifically with the phospholipid bilayer inducing membrane permeabilization and disruption [31], can also bind preferentially to a number of receptor-like membrane proteins [43]. It is conceivable that at low aggregate concentration such “ligand” behaviour may be prevalent with respect to a more massive membrane disrupting effect. RAGE is one of the most studied membrane proteins involved in amyloid binding [35–37] and, in many cases, its activation by different ligands can result in NADPH oxidase-dependent ROS production [38]. Moreover, RAGE expression and activation are characteristic hallmarks of diabetes [44,45]. Given that RAGE expression can be increased by its ligands and by amyloid deposits, and that in BV-2 cells hIAPP aggregates bind RAGE with a K_d of about 68 nM

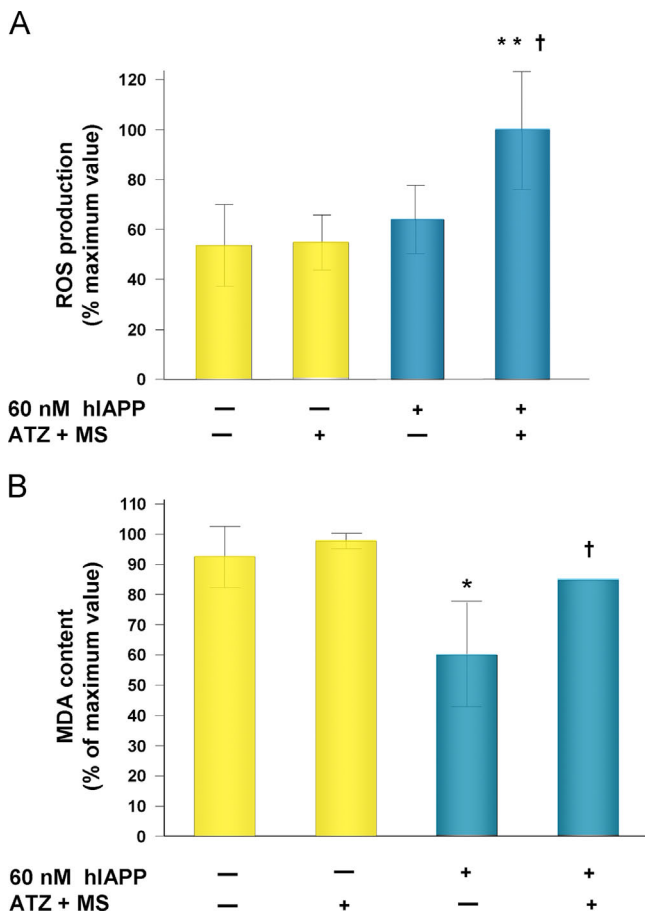


Fig. 6. ROS level and lipid peroxidation in RIN-5F cells exposed to hIAPP aggregates. ROS level (A) and lipid peroxidation, assessed as MDA content (B) in RIN-5F cells pre-treated or not for 1 h with 20 mM ATZ and 7.0 mM MS before a 3 h exposure to 60 nM hIAPP or to vehicle. Data are expressed as percentage of maximum value for each experiment and presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ Vs vehicle-treated cells; † $p < 0.05$ Vs hIAPP-treated cells. $N = 5$, separate experiments. Yellow bars, vehicle-treated cells; blue bars, hIAPP-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[37,46], we checked whether the same was true even at our experimental conditions. Actually, we observed a significant increase of RAGE level in cells exposed for 3.0 h to 60 nM hIAPP aggregates that matched that of NADPH oxidase expression and activity and was abrogated by pre-treatment with RAGE-blocking antibodies, supporting the hypothesis that it depends on oligomer-RAGE interaction.

Our novel finding was that hIAPP aggregate-mediated NADPH oxidase activation (*via* RAGE) was not accompanied by a significant build-up of ROS and the level of lipid peroxidation was even significantly reduced, although the same hIAPP aggregates were toxic when administered to cells for a longer time period (24 h) [21]. These data were quite unexpected, considering that oxidative stress is a shared biochemical modification in cells exposed to toxic amyloids [7,47], and that pancreatic β -cells in general, particularly RIN-5F cells, are reported to possess scarce antioxidant defences [8,9,48]. An explanation of our surprising results came from the analysis of the enzymatic antioxidant defences of the cells at our experimental conditions. In fact, in oligomer-exposed cells we observed a significant up regulation of CAT and GPx activities, similar to that previously reported in other biological systems following NADPH oxidase activation [26,49]. In our experimental model CAT and GPx activity upregulation matched a parallel increase of their expression. At this stage our knowledge

of the mechanism by which the observed upregulation of protein expression does occur is incomplete; however, we showed it was dependent on both RAGE engagement and NADPH oxidase activation, since it was abrogated by cell pre-treatment with either blocking anti-RAGE antibodies or DPI. Accordingly, a signalling role of NADPH oxidase-mediated ROS production can be hypothesised. In fact, among ROS producing systems, NADPH oxidases may be considered unique in that they generate ROS in a highly regulated manner, whereas all other enzymatic sources generate ROS as a by-product of the enzyme activity [17,50]. Our results extend previous findings obtained in other cells (cardiomyocytes, cancer cells) supporting the beneficial effects of low/moderate concentrations of ROS, that can be seen as a hormetic stimulus [50,51]. Hormesis can be defined as the phenomenon by which a stressor which is toxic at high doses, at low doses becomes protective by stimulating cell defence responses. Our data suggest that the response of our β -cell model to low amounts of hIAPP aggregates present for short time periods displays at least some features of hormesis. In fact, while high doses of hIAPP aggregates (10–30 μ M) inhibit antioxidant enzyme activity and are toxic to the exposed cells also by increasing the oxidative stress to unsustainable levels [12,14], low doses of hIAPP aggregates administered for a limited time period to the cells may turn out to be protective by upregulating the activity of the same antioxidant enzymes and reducing the extent of lipoperoxidation.

Growing interest is being paid to hormesis in the context of amyloid diseases. For example, it has been observed that A β amyloid aggregates, whose accumulation in the brain is considered one of the main hallmarks of Alzheimer's disease, when infused in the mouse hippocampus at picomolar-low nanomolar level increase acetylcholine production [52,53] stimulate long-term potentiation and improve cognitive performance [53]. Similarly, non-harmful levels of ER stress following moderate exposure to amyloid aggregates protect against experimental Parkinson disease [54]. Finally, for what type 2 diabetes is concerned, hormesis has been evoked to explain the resistance of some subjects to diabetes-promoting lifestyle factors [55], the anti-diabetic effect of physical exercise [56] and, in general, the variable rates of β -cell deterioration along the transition from the adaptive/compensatory stage to the terminal, severely decompensated, phase of the disease.

Our findings provide new insights into the pathophysiological processes involved in the development of type 2 diabetes supporting that, in the pre-diabetic compensatory phase, a low concentration of hIAPP amyloid aggregates may enhance the enzymatic antioxidant defences through a RAGE-Nox1-mediated pathway. Anyway, due to the multifactorial character of type 2 diabetes, care should be taken in the interpretation of these results. A recent review has outlined the protective role of mild oxidative stress during the early stages of type 2 diabetes, focusing on the production of 4-hydroxyalkenals (a by-product of lipid peroxidation) as an hormetic factor [57]. A low dose of 4-hydroxyalkenals would promote several protective responses, among which the increased expression of antioxidant enzymes *via* the Nrf 2 transcription factor [58], and the upregulation of insulin secretion *via* the activation of PPAR δ [57]. This hormetic response could be counteracted by the one that we describe, since low doses of hIAPP aggregates reduce lipid peroxidation in the case the two phenomena are contemporary, whereas the two hormetic responses could vicariate each other if they occur sequentially during the development of the disease. This matter deserves further investigation in models closely mimicking the complexity of the disease.

Our results shed new light on the reported presence of hIAPP aggregates also in non-diabetic human pancreas [59] and suggest that care should be taken when thinking of an anti-amyloid therapy for the prevention of type 2 diabetes, as this could

paradoxically speed up the progression of the disease. On the other side, such an approach could be appropriate when the disease has entered the decompensation phase, when the accumulation of toxic amyloid species, in addition to other diabetes-promoting factors, may overwhelm the hormetic effect resulting in a failure of the cellular defences with progression towards type 2 diabetes.

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