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Copper-mediated formation of hydrogen peroxide from the amylin peptide: A novel mechanism for degeneration of islet cells in type-2 diabetes mellitus?

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Abstract **Amyloid deposits derived from the amylin peptide accumulate within pancreatic islet β-cells in most cases of type-2 diabetes mellitus (T2Dm). Human amylin 'oligomers' are toxic to these cells. Using two different experimental techniques, we found that H_2O_2 was generated during the aggregation of human amylin into amyloid fibrils. This process was greatly stimulated by Cu(II) ions, and human amylin was retained on a copper affinity column. In contrast, rodent amylin, which is not toxic, failed to generate any H_2O_2 and did not interact with copper. We conclude that the formation of H_2O_2 from amylin could contribute to the progressive degeneration of islet cells in T2Dm.**

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Keywords: Type-2 diabetes; Amylin; Hydrogen peroxide; Electron spin resonance; Amplex red; Copper

1. Introduction

Type-2 diabetes mellitus (T2Dm) is an incurable disease that is characterized by hyperglycaemia arising from defects in both insulin action at responsive tissues and insulin secretion from β-cells in the pancreatic islets of Langerhans. Amyloid deposits are found in islet β-cells in up to 90% of patients with T2Dm [1]. They are derived from a peptide hormone called amylin [2] (or, alternatively, islet amyloid polypeptide [3]) that is made and co-secreted along with insulin [1]. In T2Dm, the levels of amylin are raised in parallel with the increased demand for insulin, and this is thought to induce concentration-dependent amylin aggregation [1]. Islet amyloid formation is associated with reduced β-cell mass [4] and human amylin 'oligomers' (small, soluble aggregates) are toxic to cultured islet cells [5,6], suggesting that they could contribute to progressive islet β-cell failure. Amylin oligomers can disrupt membranes [7,8] and inflict oxidative damage to cells [9], but the precise molecular mechanisms responsible for these effects have not been established.

The Aβ peptide, which accumulates at the centre of senile plaques in the brain in Alzheimer's disease (AD), can generate H_2O_2 directly from molecular oxygen, apparently *via* electron-transfer reactions involving bound redox-active transition metal ions (Fe and Cu) [10]. H_2O_2 is itself toxic to cells, but, if formed in the vicinity of redox-active metal ions, such as Fe(II), is readily converted into hydroxyl radicals, *via* Fenton's reaction. These highly reactive radicals can induce damaging oxidation, cell toxicity and cell death.

Here, we show that the human amylin peptide also has the ability to generate H_2O_2 during amyloid fibril formation in vitro, and that this process is greatly stimulated by Cu(II) ions.

2. Materials and methods

2.1. Synthetic peptides

Human amylin (amide) (hA) (Fig. 1) was purchased from American Peptide Company, Sunnyvale, CA, USA. Its purity was 93.3% as determined by high-performance liquid chromatography mass spectroscopic analysis. The equivalent rodent amylin peptide (rA) (Fig. 1) was purchased from the same source (purity 99%).

The Aβ(1–40) and Aβ(1–42) peptides were synthesised on a Milligen 9050 peptide synthesiser as described in detail elsewhere [11]. The control peptides Aβ(40–1) (reverse) and Aβ(1–40)Met^{35}Nle were from Biosource International (Belgium).

2.2. Amplex Red assay

Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) is a non-fluorescent compound that is oxidised by H_2O_2 in the presence of horseradish peroxidase (HRP) to yield the highly fluorescent product, resorufin [12]. Both the Aβ and amylin peptides were incubated at 100 μM in 50 mM Na phosphate, 0.14 M NaCl, pH 7.4 (PBS) at 37 °C for selected time periods before testing for H_2O_2 formation. For the Amplex Red assays, a 10 mM stock solution of Amplex Red was prepared in

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; AFM, atomic force microscopy; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, DMPO hydroxyl radical adduct; DMSO, dimethyl sulfoxide; EM, electron microscopy; ESR, electron spin resonance; hA, human amylin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; rA, rodent amylin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T2Dm, type-2 diabetes mellitus

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1 10 20 30 37
hA KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH_2
rA KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY-NH_2

Fig. 1. Amylin peptides employed for this study. Amino acid differences between human amylin (hA) and rodent amylin (rA) are underlined.

dimethyl sulfoxide (DMSO) and a HRP stock solution (10 U/ml) was prepared in phosphate-buffered saline (PBS) (both stored at −20 °C). To produce the Amplex Red working solution, the stock HRP was diluted 1/1000 in PBS and this diluted HRP solution (100 μl) and Amplex Red stock solution (50 μl) were added to 4.85 ml of PBS. Standard curves were obtained using H_2O_2 solutions with concentrations ranging from 0.05 to 1.0 μM. For the assay, a 10 μl sample of standard H_2O_2 solution, or test sample, was added to each well of a black, flat-bottomed, 384-well microtitre plate (Nunc), in triplicate, and then 10 μl of Amplex Red working solution was added. Similar results were obtained with 50 μl samples plus 50 μl Amplex Red working solution, in 96-well plates. Fluorescence of the resorufin product was read, after 30 min incubation at room temperature, on a Victor^2 1420 plate reader, with λ_{Ex} = 544 nm and λ_{Em} = 590 nm. Solutions containing no H_2O_2 were used as a measure of background fluorescence, which was subtracted from all values.

In some experiments, catalase (1000 U/ml) was added to the peptide samples at the end of their incubation period, before they were added to the Amplex Red working solution. In others, the metal ion chelator diethylenetriaminepentaacetic acid (DETAPAC) (100 μM) was added prior to incubation of the peptide solutions.

To study the effects of metal ions, hA (100 μM) was incubated for various times at 37 °C in the presence of 10 μM of each of the following:- $CuCl_2$, $ZnCl_2$, $FeCl_3$, $AlCl_3$, $MgCl_2$; rA was incubated with 10 μM $CuCl_2$ as a control. In these experiments, HEPES buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4) was used in all treatments (including controls) instead of PBS. The samples were then tested using the Amplex Red method.

2.3. Electron spin resonance (ESR) spectroscopy

The hydroxyl radical was formed (*via* Fenton chemistry) from any H_2O_2 generated during incubation of the required peptide at 100 μM in PBS, at 37 °C and detected by ESR spectroscopy, as described previously [13–15]. The method employed the reaction of this radical with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) to form the hydroxyl radical adduct (DMPO-OH), which has a uniquely characteristic 4-line ESR spectrum with a(N) 1.50 and a(H_β) 1.46 mT [13–15]. Samples of the required peptide (50 μl), incubated both in the absence and presence of various metal ions (2 μM), were withdrawn at selected time-points for ESR analysis.

2.4. Atomic force microscopy

Samples selected for analysis by atomic force microscopy (AFM) at various time points were diluted 5 times in MQ water prior to imaging. The diluted samples (1 μl) were applied to freshly cleaved mica-covered metal sample stubs and air dried. AFM imaging was undertaken using a Multimode AFM with a Nanoscope IIIa controller in tapping mode in air with Si_3N_4 high spring constant cantilevers (TESP, Veeco, Cambridge, UK). A high-resolution scanner (E-type scanner, Veeco) was used to allow the best possible resolution of deposits at early incubation times. Five randomly chosen areas were imaged on each sample and the subsequent images were then carefully analyzed for ultrastructural differences.

2.5. Transmission electron microscopy

Samples of incubated peptides were selected for analysis by electron microscopy (EM) at various time points. They were applied to Formvar-coated nickel grids for 2 min and air dried prior to negative staining with 2% (w/v) uranyl acetate for 30 s and wicked using filter paper. The samples were air-dried and examined on a JEOL 1010 TEM.

2.6. Immobilised metal affinity chromatography

HiTrap chelating columns (Amersham Biosciences, 1 ml) were charged with either 0.1 M Cu(II) chloride or 0.1 M Fe(III) chloride and then washed with 5 ml of MQ water and 10 ml of 20 mM sodium phosphate, 0.5 M NaCl, pH 8 (metal-binding buffer) to remove excess metal ions. hA or rA (100 μg/ml in metal binding buffer) were then loaded onto the columns. The peptides were eluted by using several 'stepwise' concentrations of imidazole (from 0 to 100 mM in metal binding buffer). The fractions from the columns were analyzed by 4–20% Tris–Glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

3. Results

We used two quite independent methods in order to determine if synthetic human amylin peptide (hA), or the equivalent non-amyloidogenic and non-toxic rodent amylin peptide (rA), can generate H_2O_2 during incubation in vitro. The first method employed the dye Amplex Red [12], whereas the second method was based on our previously published ESR spectroscopy technique [13–15]. We also examined the time period during which any H_2O_2 was formed. The ultrastructure of the peptide aggregates was assessed from a study of AFM and EM images.

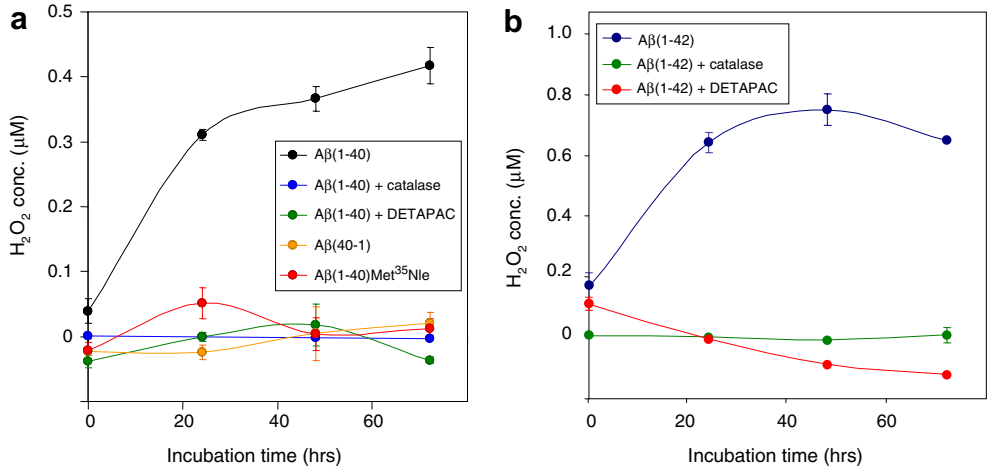

Fig. 2. Results for Aβ, employing the Amplex Red method. (a) Aβ(1–40) incubated with and without catalase or DETAPAC, compared with Aβ(40–1) and Aβ(1–40)Met^{35}Nle; (b) Aβ(1–42) incubated with and without catalase or DETAPAC.

3.1. Amplex Red assays

We initially carried out experiments with Aβ(1–40), Aβ(1–42) and related control peptides to validate the Amplex Red method. These peptides were incubated at 100 μM in PBS at 37 °C and samples were taken at various times for H_2O_2 assay. An Amplex Red signal, which increased rapidly during the early course of aggregation, was detected from both Aβ(1–40) and Aβ(1–42) (Fig. 2). Overall, these results were compatible with our published ESR data showing that H_2O_2 is generated during the early stages of Aβ aggregation [16]. Inclusion of catalase at the end of the peptide incubation period blocked the Amplex Red signal, as did the presence during incubation of the metal-ion chelator DETAPAC. Aβ(1–40)Met^{35}Nle and Aβ(40–1) (reverse peptide), both of which have been shown previously not to generate H_2O_2 [17], failed to produce any signal in the Amplex Red assay.

We then proceeded to measure the levels of H_2O_2 in similarly incubated solutions of hA and rA. In the case of hA, H_2O_2 formation was detected during the course of peptide aggregation, reaching a maximum at around 72 h incubation time, before declining to background levels at longer incubation times (Fig. 3a). In contrast no H_2O_2 was detected during the incubation of rA. The rate of decay of the H_2O_2 self-generated from hA, and hence its stability, depends upon two factors. Firstly, direct reaction with the peptide and, secondly, on the availability of metal ions to convert it to hydroxyl radicals. A classic example of the differences in this rate of decay can be found when comparing H_2O_2 levels generated by Aβ (slow decay) with those from ABri (rapid decay) [16].

Freshly dissolved hA peptide showed no clear structures (Fig. 4a) but analysis of EM and AFM images obtained during the early stages of H_2O_2 formation (3–6 h) revealed the presence of numerous short 'protofibrils' (Fig. 4b and c) whereas samples examined after prolonged incubation periods (24–72 h) revealed the presence of longer amyloid fibrils (Fig. 4d). The peroxide was, therefore, generated during amyloid fibril formation. The AFM images of the protofibrils formed from hA (Fig. 4c) were similar in appearance to those observed with Aβ [16]. When hA was incubated in the presence of various metal ions, only Cu(II) stimulated the formation of H_2O_2 to any great extent (Fig. 3b). In the presence of Cu(II), the Amplex Red signal from hA again reached a peak at around 72 h incubation time, but there was a 6-fold increase in H_2O_2 levels compared with those obtained from hA alone. The ultrastructural appearance of the hA aggregates formed during incubation, or the aggregation time, did not appear to be markedly changed by the presence of Cu(II) ions (Fig. 4e and f). It should be noted that no distinct fibrils were observed with rA incubated with or without Cu(II) ions.

In all of these experiments, any positive signal from hA was always blocked by the addition of catalase at the end of the peptide incubation period (confirming the detection of H_2O_2) or by the inclusion of DETAPAC during peptide incubation (suggesting that metal ions are involved) (Fig. 3a). The presence of Cu(II) ions during the incubation of rA failed to stimulate the formation of H_2O_2 (Fig. 3b).

3.2. ESR measurements

For the ESR method, hA was incubated (as before) in the presence or absence of various metal ions, and any H_2O_2 formed was detected by its conversion into hydroxyl radicals, which were trapped with DMPO [13–15]. A weak 4-line spectrum corresponding to the hydroxyl radical adduct was often observed without added metal ions, but this was sometimes absent altogether (Fig. 5a). However, the intensity of this spectrum was greatly enhanced when hA was co-incubated with Cu(II) ions (Fig. 5b). All of the ESR spectra obtained from hA could be blocked with catalase (Fig. 5c) or DETAPAC (Fig. 5d) and no spectra were observed under any conditions from rA (Fig. 5e).

3.3. Copper ion binding to hA

hA was retained on a HiTrap metal-chelating column charged with Cu(II) ions and could be subsequently eluted with 25 mM (but not 10 mM) imidazole (Fig. 6a). This peptide failed to bind to a similar column charged with Fe(III) ions (Fig. 6b). Furthermore, rA did not bind to either of these metal-loaded Hitrap columns (Fig. 6c).

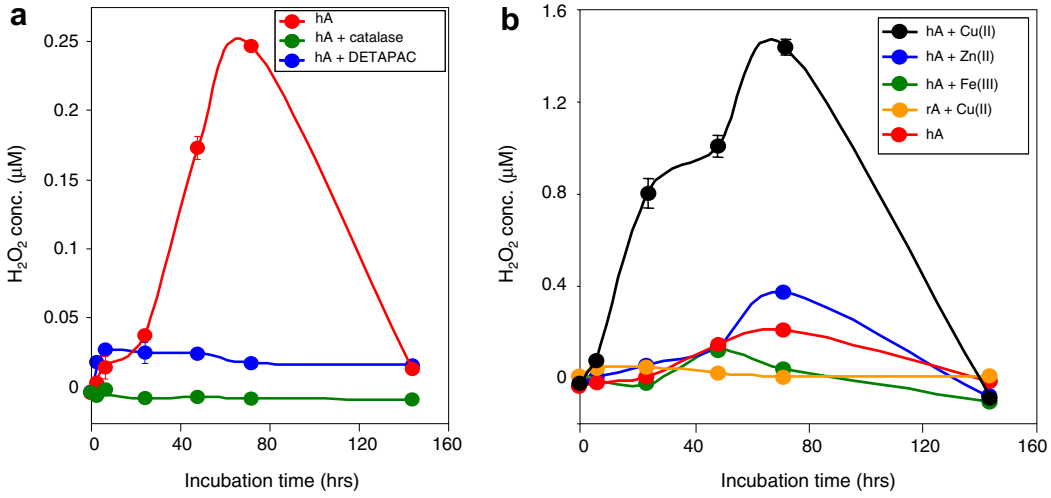

Fig. 3. Results for amylin, employing the Amplex Red method. (a) hA, with and without catalase or DETAPAC; (b) effects of metal ions (10 μM) on hA and rA. In addition to those shown, Al(III) and Mn(II) had no significant effect on the Amplex Red signal from hA.

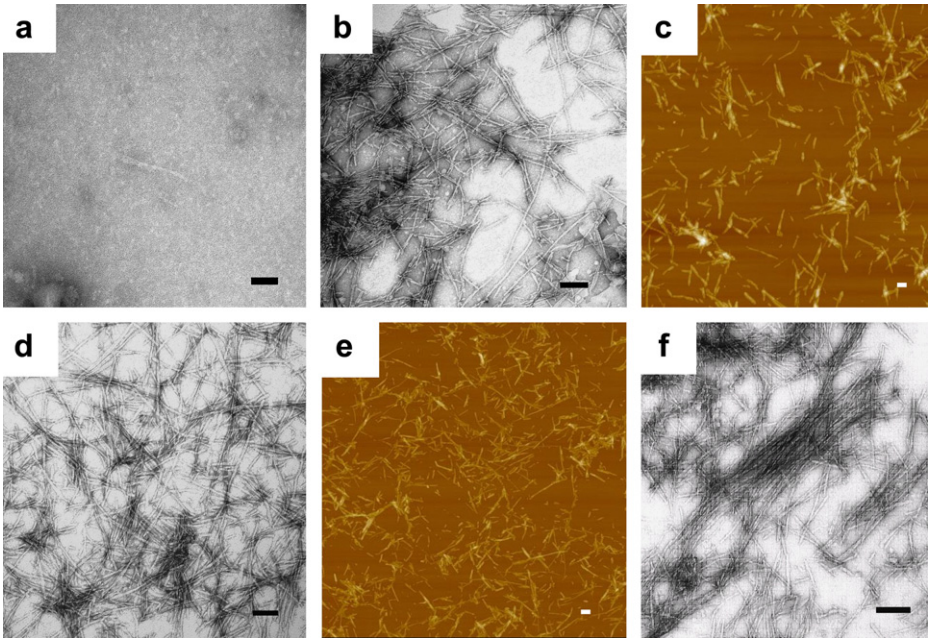

Fig. 4. Ultrastructural appearance of hA aggregates. (a) EM image at zero incubation time; (b) EM image of 'protofibrils' at 6 h incubation; (c) AFM image of 'protofibrils' at 6 h incubation; (d) EM image of fully-formed amyloid fibrils at 72 h; (e) AFM image at 3 h incubation, in the presence of Cu(II); (f) EM image at 72 h incubation, in the presence of Cu(II).

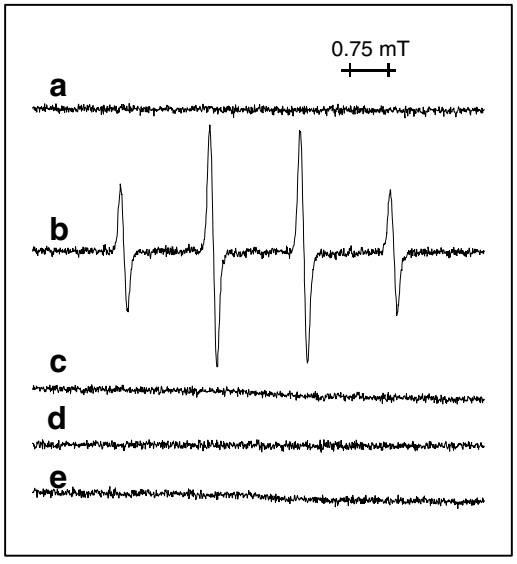

Fig. 5. ESR spectra recorded from amylin peptides. (a) hA; (b) is (a) incubated in the presence of Cu(II) (2 μM); (c) is (b) incubated in the presence of catalase; (d) is (b) incubated in the presence of DETAPAC; (e) rA incubated in the presence of Cu(II) (2 μM). A control experiment containing no peptide gave spectra similar to those shown in (a), (c), (d) and (e).

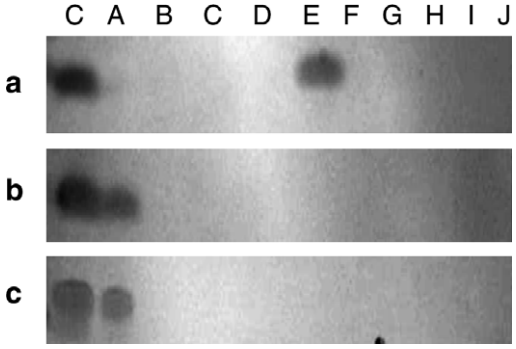

Fig. 6. Elution of amylin from metal-loaded HiTrap columns. (a) hA binds to Cu(II) and is eluted with 25 mM imidazole: C = hA control; A,B = 0 mM imidazole; C,D = 10 mM imidazole; E,F = 25 mM imidazole; G,H = 50 mM imidazole; I,J = 100 mM imidazole. (b) hA fails to bind to Fe(III) and is eluted with the first wash: lanes as before; (c) rA fails to bind to Cu(II): lanes as before.

4. Discussion

Although it has been established previously that exposure to the hA peptide increases H_2O_2 levels in cultured cells [18], our results, employing two completely independent techniques (i.e., Amplex Red and ESR spectroscopy), demonstrate, for the first time, that H_2O_2 is generated directly during the aggregation of hA in vitro, and also that the formation of H_2O_2 in this way is selectively stimulated by Cu(II) ions. The accumulation of H_2O_2 was confirmed by the experiments with catalase, which blocked both of the Amplex Red and ESR signals. Similar experiments with DETAPAC suggest the involvement of metal ions, even when these are not added externally to the peptide.

The demonstration of an interaction between hA and the copper-loaded HiTrap columns suggests a mechanism for the generation of hydrogen peroxide involving the binding of Cu(II) ions to hA. This would potentially result in the formation of a redox-active complex. The donation of electrons from the peptide to Cu(II) ions would result in the formation of a Cu(I)-peptide complex, which could also be formed by the direct binding of the peptide to Cu(I) ions. The Cu(I)-complex could stimulate the formation of H_2O_2 from O_2 and result in

a consequent return to the Cu(II) oxidation state. Subsequent cycling of this redox system could occur, resulting in the generation of hydroxyl radicals from H_2O_2 via Fenton chemistry.

The formation of H_2O_2 from hA by this type of mechanism could explain the well-established toxicity of this peptide towards cultured islet cells. This would be in accord with previous reports that the cytotoxicity induced by hA involves oxidative damage [9] and can be reduced by anti-oxidants [19]. The ready formation of hydroxyl radicals from H_2O_2 in the presence of redox-active transition metal ions could also contribute towards in vivo damage to the pancreas in T2Dm, for which there is extensive evidence for the involvement of reactive oxygen species [1].

Our results also add weight to the emerging hypothesis that oxidative damage during early oligomer formation could represent a common mechanism of toxicity for many amyloidogenic proteins and peptides [8,13–17,20]. This hypothesis is further supported by the fact that rA, which is not toxic to cells [21,22], failed to generate any H_2O_2 in our experiments, presumably due to its inability to aggregate and/or to interact with copper ions.

A possible role for copper ions in T2Dm has already been implied by the finding that the level of copper is significantly higher (while those of zinc and magnesium are lower) in serum from diabetic patients than controls [23]. This suggest that copper could play an important role in the pathogenesis of T2Dm, possibly including the facilitation of H_2O_2 generation from amylin, and, ultimately in the degeneration and death of pancreatic islet cells. Our data provide a new rationale and added impetus for the clinical use of copper chelators in T2Dm [24] as well as opening up additional avenues of research into the etiology, pathogenesis and treatment of this disease.

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