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Vegetable Oil-derived 'Hydroxynonenal' Causes Diverse Cell Death Possibly Leading to Alzheimer's and Related Lifestyle Diseases

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Abstract

Objective: The real culprit of Alzheimer's disease remains unelucidated for more than a century. Since Alzheimer's disease is often associated with lifestyle diseases such as type 2 diabetes, there should be a common causative factor. To elucidate this, we focused on 'Hydroxynonenal' that is generated during deep frying of ω-6 Polyunsaturated Fatty Acids (PUFAs).

Methods: Monkeys after consecutive injections of the synthetic Hydroxynonenal were histologically studied to determine, whether it can induce cell degeneration/death in the brain, liver and pancreas.

Results: In all of the five monkeys injected, hippocampal neurons, hepatocytes and β cells after Hydroxynonenal injections revealed similar microcystic degeneration and scattered cell death by light microscopy. By electron microscopy, degenerating cells generally showed lysosomal permeabilization or rupture, electron-luscent cytoplasm, nuclear dissolution, membrane disruption, mitochondrial injury and accumulation of autophagosomes containing cell debris. The number of vivid lysosomes were remarkably decreased, compared to the controls.

Conclusion: Targeting 'Hydroxynonenal' would help elucidate the pathogenesis of not only Alzheimer's disease but also related lifestyle diseases. Since ω-6 PUFAs can induce both GPR40 activation leading to calpain activation and intrinsic Hydroxynonenal generation leading to Hsp70.1 carbonylation, calpain-mediated cleavage of carbonylated Hsp70.1 was thought to disturb lysosomal membrane integrity to induce programmed cell death.

Keywords: Calpain-cathepsin hypothesis; Cell death; Hsp70.1; Lysosome; ω-6 PUFA

Abbreviations: NAFLD: Non-alcoholic Fatty Liver Diseases; NASH: Non-alcoholic Steatohepatitis; PUFAs: Polyunsaturated Fatty Acids; Hsp70.1: Heat-Shock Protein 70.1; H-E: Hematoxylin-Eosin; CA1: Cornu-*Ammonis* 1; ER: Endoplasmic Reticulum; LC-MS/MS: liquid Chromatography-Mass Spectrometry/Mass Spectrometry; GPR40: G Protein-coupled Receptor 40; LDL: Low-Density Lipoprotein

Introduction

Lifestyle-related diseases (lifestyle diseases) are defined as diseases linked with the way people live their life. Representative diseases that are affected by lifestyle are chronic diseases such as Alzheimer's disease, Nonalcoholic Fatty Liver disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH), type 2 diabetes, obesity, arteriosclerosis, hypertension, etc. These diseases are commonly caused by unhealthy food habits and overeating, lack of physical exercise, alcohol and drug abuse, smoking, addiction to mobile phones, etc. The onset of lifestyle disease is insidious, taking years to develop and once encountered they do not lend themselves easily to cure. Since etiology of lifestyle diseases is complex and varies between individuals, it is difficult to identify the most common and essential causative factor, especially if it's relative effect is weak and many years are needed for both the adverse health effects and the disease progression.

Oxidative stress is known to promote cellular dysfunction by inducing DNA damage, protein modification and lipid peroxidation. The latter is the oxidative deterioration of Polyunsaturated Fatty Acids (PUFAs) which contains two or more carbon-carbon double bonds (C=C). Hydroxyl radicals (●OH) readily initiate peroxidation of fatty acids, lipoproteins and cell membranes. Following lipid peroxidation, Hydroxynonenal and malondialdehyde are the most abundant aldehydes produced, while acrolein is the most reactive [1-3]. During the last decade, one of the most abundant lipid peroxidization products, 'Hydroxynonenal' has been shown to be involved in diverse pathologies such as neurodegenerative diseases and metabolic diseases [4-7]. For example, the concentration of Hydroxynonenal-protein adducts has been shown to be highly elevated in human plasma/serum obtained from obese subjects, compared to the lean subjects [4]. In Alzheimer's disease, Hydroxynonenal-adducts to neurofilaments were found and high levels of Hydroxynonenal were detected within amyloid β plaques and in the cerebrospinal fluid of patients with Alzheimer's disease [5,6]. In addition, Hydroxynonenal may trigger β cell apoptosis, induce glucose intolerance and develop type 2 diabetes [7].

A diet of junk food, overeating of vegetable oils and lack of physical activity should be altogether responsible for the intrinsic generation of

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reactive oxygen species and Hydroxynonenal related to the oxidization of PUFA in the cell membrane. One of the difficulties in modeling lifestyle diseases is reconciling acute *in-vitro* models with the gradual development of the actual pathology. Oxidative damage *in vivo* slowly accumulates over years until a threshold was passed at which pathology becomes overt for the slow progression of the disease. The main objective of this study is to determine whether 'Hydroxynonenal' can induce cell degeneration/death in the brain, liver and pancreas, with a particular emphasis on the role of heat-shock protein70.1 (Hsp70.1) known to have dual functions as a chaperone protein and lysosomal stabilizer. Because of its chemical reactivity of oxidizing Hsp70.1, Hydroxynonenal can exert pleiotropic effects particularly in the lysosomal cell death by inducing membrane disruption [8-10]. Since lysosomal membrane permeabilization/rupture and mitochondrial failure, the hallmarks of cell degeneration/death for diverse lifestyle diseases, can be observed only by electron microscopy, here, we focused on not only light microscopic but also electron microscopic features of Hydroxynonenal-induced cell degeneration/death which occurred in the brain, liver and pancreas.

In this study, the following two questions were addressed:

1) Does consecutive but sub-lethal injections of the synthetic Hydroxynonenal cause, *in vivo*, lysosomal permeabilization/rupture and mitochondrial damages leading to the cell degeneration and death in the brain (especially, lesions closely related with the occurrence of Alzheimer's disease and obesity), liver and pancreas?

2) The authors formerly formulated the 'calpain-cathepsin hypothesis' as a mechanism of neuronal death after transient brain ischemia [11]. Calpain-mediated cleavage of carbonylated Hsp 70.1 facilitates lysosomal rupture with the resultant spillage of cathepsins [12-14]. Can this hypothesis explain also the mechanism of diverse cell degeneration/death being induced by Hydroxynonenal?

Materials and Methods

Animals

Nine young (4~5 years) monkeys (Macaca fuscata) were supplied by National Bio-Resource Project (NBRP) "Japanese monkey" (National Institute for Physiological Sciences, Okazaki, Japan). Upon arrival, they were fed ~100g × 2/day of normal (nonpurified) diet for 1 year to facilitate acclimation. At 5~6 years of age, monkeys with body weight 5~7 Kg were randomly divided into two groups of the sham-operated controls (n=4) and those undergoing Hydroxynonenal injections (n=5). Intravenous injections of 5 mg/week of synthetic Hydroxynonenal (Cayman Chemical, Michigan, USA) were done for 24 weeks. Such doses and serial injections were designed to grossly mimic blood concentrations of Hydroxynonenal in humans around 60's or the average concentrations (20 μmol/L) in patients with Alzheimer's disease [15]. The Kanazawa University Animal Care and Use Committee approved the protocol (AP-153613). All experimental procedures were strictly in accordance with the guidelines for the care and use of laboratory animals of both Kanazawa University and the National Institutes of Health guide for the care and use of laboratory animals.

Tissue collection

At 24 weeks after the initial injection with the accumulative Hydroxynonenal dose of 120 mg, the monkeys were deeply anesthetized with GOF (80% nitrogen, 20% oxygen and 1.5% halothane), then, during the perfusion of 500 mL saline through the left ventricle, the total blood was removed from the right atrium by cutting the atrial appendage. Then, 500 mL of 4% paraformaldehyde were perfused through the left ventricle and the brain (hippocampus, hypothalamus, precuneus and the posterior cingulate gyrus), liver (areas within and around the scar) and pancreas were resected and fixed in either i) 4% paraformaldehyde for 2 weeks for light microscopy or ii) 2.5% glutaraldehyde for 2 h for electron microscopy.

Histological and ultrastructural analyses

The tissues after fixation with 4% paraformaldehyde were embedded in paraffin and 5 μm sections were routinely stained for Hematoxylin-Eosin (H-E). The remaining tissues after fixation with 2.5% glutaraldehyde were post-fixed with 1% OsO_4 for 1 h. They were dehydrated with graded acetone, embedded in resin (Quetol 812, Nisshin EM Co. Tokyo) and thin sections were made. After trimming with 0.5% toluidine blue-stained sections, the ultrathin (70 nm) sections of selected areas were stained with uranyl acetate (15 min) and lead citrate (3 min) and observed by the electron microscope (JEM-1400 Plus, JEOL Ltd., Tokyo).

Results

Neuronal loss in the hippocampus, precuneus and the posterior cingulate gyrus are known to be closely related with the progression of Alzheimer's disease, while that of arcuate nucleus in the hypothalamus is related with failure of the appetite (energy) and body weight control, leading to obesity [16,17]. In addition, cell degeneration/death of hepatocytes is related with chronic liver diseases such as NAFLD and NASH, while that of pancreatic β cells and δ cells is related with the occurrence of type 2 diabetes [18]. Accordingly, the author focused on the damage of these cells after Hydroxynonenal injections and found that all of the five monkeys after Hydroxynonenal injections revealed membrane disruption as well as lysosomal and mitochondrial disruption in the brain, liver and pancreas. Each cells showed microcystic degeneration with cytoplasmic shrinkage and dissolution of the nuclear chromatin. Although focal cell death was seen in the brain, liver and pancreas, neither apoptosis nor condensation of the nuclear chromatin were observed.

Brain

H-E staining showed that the CA1 neurons were the most vulnerable among the four brain regions studied. The control CA1

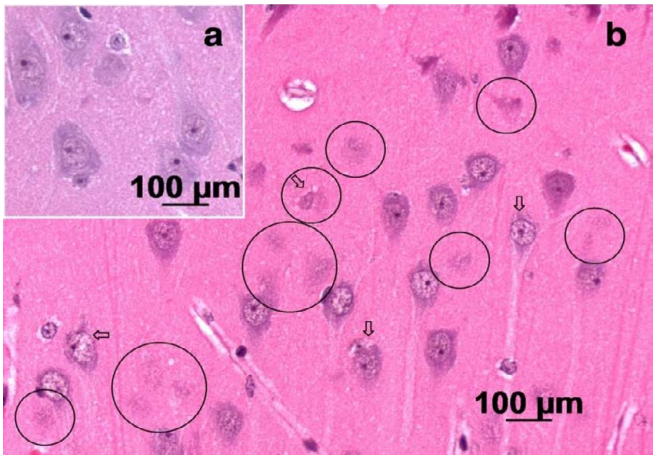

Figure 1: Compared to the control CA1 (1a), the hippocampal CA1 sector after Hydroxynonenal injections shows scattered neuronal death (circles). The degenerating CA1 neurons were characterized by intracytoplasmic microcysts (open arrows) (1b), decreased staining indicating loss of organella and shrinkage of the cell body. H-E staining.

neurons generally had large, pyramidal cytoplasm and a round nucleus with vesicular distribution of heterochromatin (Figure 1a). After Hydroxynonenal injections, however, many CA1 neurons displayed mild but distinct shrinkage of the cell body with microcystic degeneration of the cytoplasm (Figures 1b and 2b, open arrows). Some neurons showed a remarkable dissolution of the cytoplasm and the nuclear chromatin, which is compatible with necrotic cell death (Figures 1b, circles). Consistent with the light microscopic findings, electron microscopic observations of the degenerating CA1 neurons displayed frank membrane disruption and lysis of cell organelles (Figures 2b). They showed a marked dissolution of the Endoplasmic Reticulum (ER) and mitochondria, which was associated with the significant decrease of lysosomes, compared to the control neuron (Figures 2). In addition, lysosomes of the degenerating CA1 neurons showed partial sprout of the limiting membrane with forming daughter lysosomes (Figure 3a, arrows). Although rare, spillage of the lysosomal content was confirmed in the CA1 neuron (Figure 3b, star, arrows). The nucleus showed dissolution of the chromatin, so apoptotic bodies were not observed. The neurons of the arcuate nucleus in the hypothalamus and precuneus showed similar scattered neuronal death with microcystic degeneration, dissolution of organella and permeabilization of lysosomes (Figure 4). These degenerations were observed also in the posterior cingulate gyrus, although less remarkable.

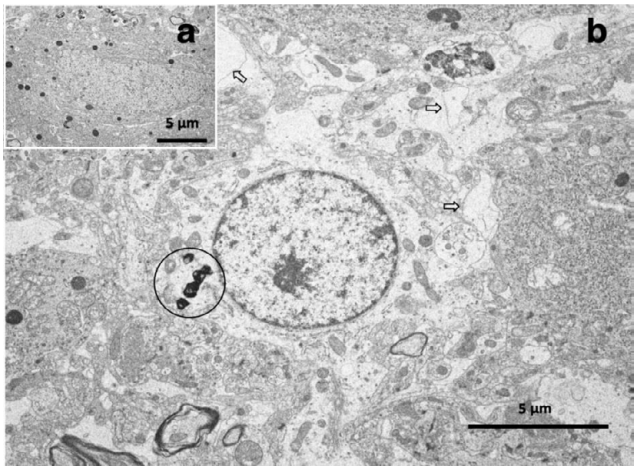

Figure 2: By the electron microscopic observation, the degenerating CA1 neuron shows clear cytoplasm with dissolution of rough ER and a complete loss of lysosomes (2b). Compared to the control CA1 neurons containing numerous lysosomes (2a), the degenerating neuron has no vivid lysosomes despite a few dense bodies which might be a terminal feature of autolysosomes containing undigested materials (2b, circle). The lysosomal loss is presumably due to their rupture as shown in figure 3b. This is consistent with degradation of abundant cell debris such as damaged organella, membrane, mitochondria, etc. The degenerating neuron is also characterized by many microcysts (2b, open arrows) in the cytoplasm as seen in figure 1b. Uranyl and lead staining.

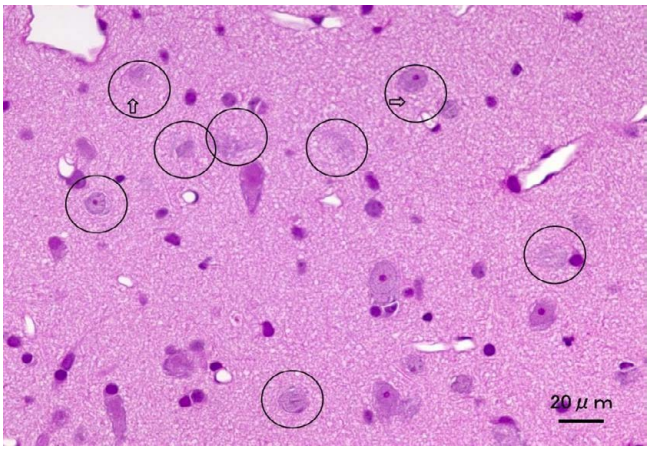

Figure 4: Neurons of the arcuate nucleus in the hypothalamus show a remarkable degeneration (circles) with microcystic changes (open arrows) and dissolution of the nuclear chromatin after Hydroxynonenal injections. The degeneration pattern of hypothalamic neurons was essentially the same with that of the hippocampus (figure 1b), precuneus, and the posterior cingulate gyrus, although the extent of degeneration was the most severe in the hippocampal CA1 sector. H-E staining.

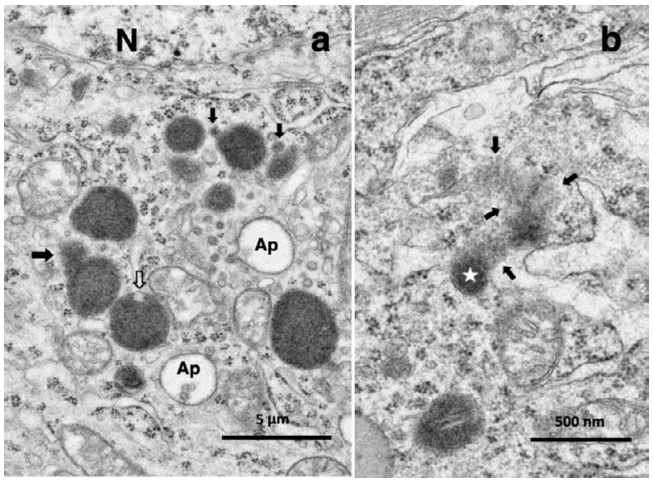

Figure 3: Lysosomes of the degenerating CA1 neuron show partial sprout of the limiting membrane, forming daughter lysosomes (3a, arrows). This is distinct from micro autophagy (3a, open arrow). In addition, spillage of the content from the lysosome (3b, arrows) was observed. Presumably, after the spillage of the total content, the lysosome (star) will disappear, and the CA1 neuron would eventually show a similar feature to figure 2b. N: Nucleus, Ap: Autophagosome. Uranyl and lead staining.

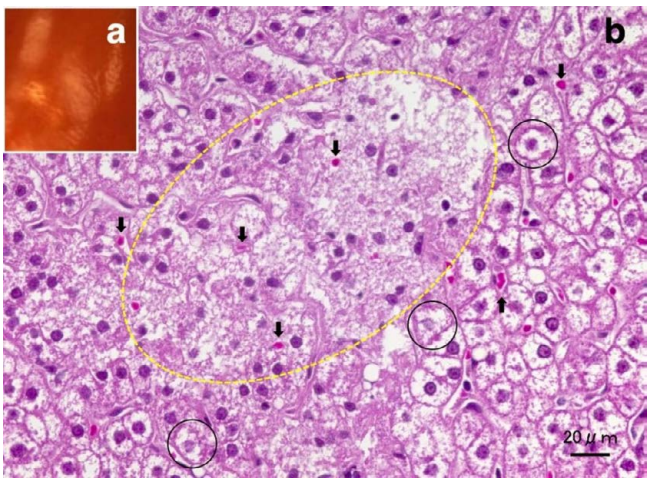

Figure 5: The liver after Hydroxynonenal injections shows a mosaic pattern of discoloring or scar formation at the surface (5a) which was microscopically confirmed to be a marked degeneration and focal cell death of hepatocytes (5b, yellow circle). The former was balloonings with dissolution of cell organelles, while the latter was necrosis with loss/decrease of cell membranes, organelles, and nuclear chromatin (5b, black circles). Not infrequently, Mallory-Denk body-like acidic structures (5b, arrows) were observed. H-E staining.

Liver

At autopsy 6 months after Hydroxynonenal injections, among the three organs studied, only the liver showed a macroscopic change; a mosaic pattern of discoloring or scar formation at the surface (Figure 5a). The discolored portion was microscopically characterized by the diffuse loss of columnar structures in the lobule, showing distinct hepatocyte injury (Figure 5b). It comprised of areas with ballooned hepatocytes (microvesicular steatosis, black circles) or necrotic hepatocellular injury (yellow circle) (Figure 5b). The ballooned hepatocytes were especially seen around the central vein or diffusely within the lobule, while the necrotic hepatocytes were seen predominantly in the periportal area. Ballooned hepatocytes had distinctive rarefied, translucent cytoplasm that is irregularly stranded and clumped (Figure 5b, black circles). The cytoplasm contained coarsely granular particles which were thought to be membrane debris mainly comprising of lipid components. Small lipid droplets were observed within the degenerating hepatocytes, being released after the cell loss (Figure 6, asterisks). The necrotic and pre-necrotic hepatocytes showed eosinophilic or amorphous cytoplasm with an almost complete loss or marked disruption of organella and nuclear chromatin (Figure 5b, black circles). Such histological features were compatible with the characteristics of coagulation necrosis. The severely degenerating (pre-necrotic) hepatocytes prior to the coagulation necrosis infrequently formed Malloly-Denk body-like acidophilic cytoplasmic inclusions (Figures 5b and 6a, arrows) as observed in NASH.

The most common feature of degenerating hepatocytes was microcystic degenerations that could be observed at the high-power observation (Figure 6a, circles). Such microcystic spaces showed a radiated distribution around the disrupted nucleus. Some micro cysts looked empty while others appeared to contain tiny cell debris-like, membranous, or eosinophilic amorphous material. Electron microscopic observation disclosed that these were not the exact microcysts but the virtual space. Most of the mitochondria showed disruption of crista and double membrane, compared to the control

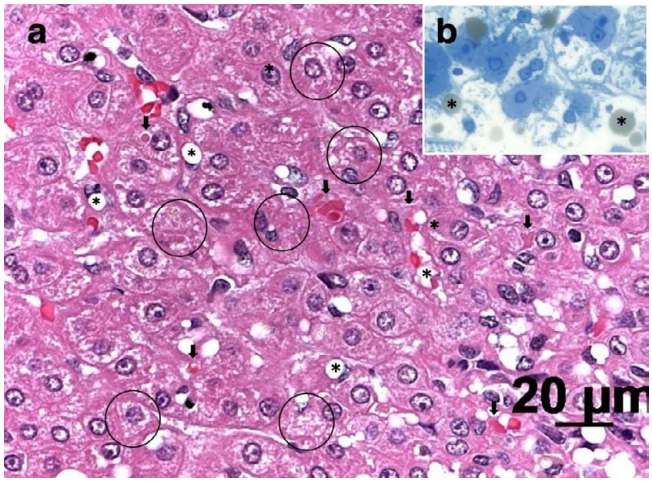

Figure 6: Degenerating hepatocytes after Hydroxynonenal injections show microcystic degeneration of the cytoplasm (6a, circles). Some microcysts appear to be empty while others contain eosinophilic cell debris. Degenerating hepatocytes contain numerous small lipid droplets (6a, asterisks) and Mallory-Denk body-like acidic inclusions (6a, arrows). The lipid droplets appeared empty on the single fixation followed by ethanol dehydration for light microscopy, but remained as yellow droplets (6b, asterisks) after the double fixation for electron microscopy. Please, note such lipid droplets are formed within the degenerating hepatocytes, and are released outside after the cell loss. a, H-E staining; b, Toluidine-blue staining.

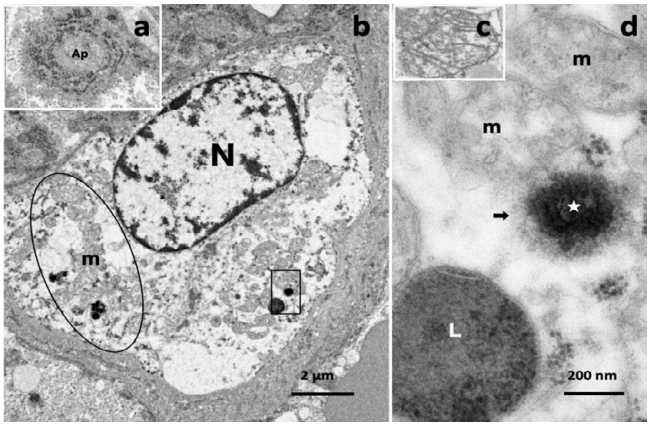

Figure 7: The degenerating hepatocyte ultrastructurally shows a clear cytoplasm with a marked dissolution of mitochondria (7b, circle, m, 7d, m), and a remarkable decrease of the lysosomes. This was similar to the feature of the degenerating CA1 neuron (figure 2b). The clear cytoplasm was consistent with balloonings as seen in figure 5b by light microscopy. Mitochondria (7d, m) show disruption of cristae, while lysosomes show evidence of membrane permeabilization (7d, star and arrow). Please, note the marked contrast with the intact lysosome (7d, L). Compared to the control mitochondria (5c), the degenerating mitochondria (7d, m) show a marked disruption of cristae and investment with rough ER membrane forming autophagosome (7a, Ap). (Rectangle of figure 7b was magnified to figure 7d.) Uranyl and lead staining.

(Figure 7). Damaged mitochondria were often invested with the ER membrane to form autophagosome (Figure 7a, Ap). The plasma membranes of the degenerating hepatocytes appeared to be disrupted, but the cell boundary was distinct because of the remarkable thickening of basement membranes (Figure 7b). Lysosomes showed a remarkable decrease as seen in the degenerating CA1 neurons and the remaining lysosome showed evidence of the membrane permeabilization, showing a marked contrast to the intact lysosome (Figure 7b).

Pancreas

Compared to the normal controls, the pancreas after Hydroxynonenal injections showed scattered cell degeneration/death within the Langerhans islet, which was also characterized by microcystic changes of the cytoplasm and dissolution of the nuclear chromatin (Figure 8, circles). Some micro cysts appeared to be empty, while others were filled with eosinophilic material. The microcysts were ultrastructurally identified to be enlarged rough ER (Figure 8c). Light microscopic observation by H-E staining failed to identify neither the cell type of each Langerhans cells nor the type of necrotic cells. Some of acinar cells adjacent to the Langerhans islets also showed mild dissolution of the nuclear chromatin after Hydroxynonenal injections.

At the ultrastructural level, the cell type in the Langerhans islets was distinguished primarily by differences in their secretion granules. The α cell granules (glucagon) are typically slightly larger than β-cell granules (insulin). δ cell granules (somatostatin) are typically less densely stained than the granules in α- and β- cells [19]. In addition, β cells were characterized by the presence of amyloid deposits and co-secretion with insulin granules [20-22].

By the electron microscopic observation, the degenerating/necrotic Langerhans cell was identified to be β cell, because of the presence of characteristic insulin granules and amyloid microfibrils (Figures 8c, β & 9, G). The degenerating β cell showed dissolution of the nuclear chromatin, remarkably-enlarged rough ER and autophagosomes containing degenerating mitochondria (Figure 8d, Ap). The pancreas

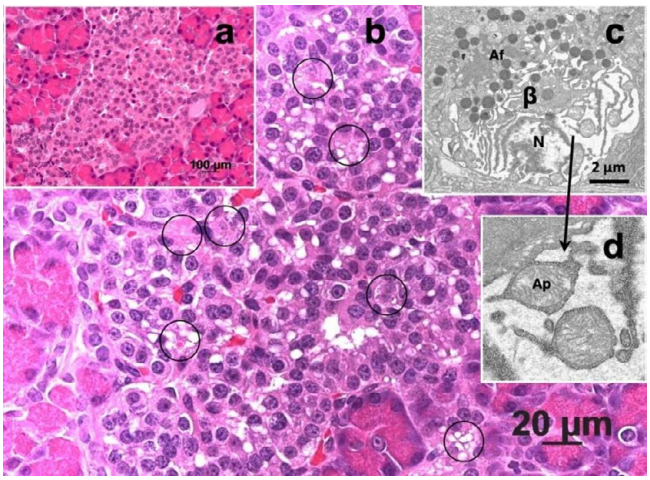

Figure 8: Compared to the Langerhans islets in the control pancreas (8a), the islet cells after hydroxynonenal injections show microcystic degeneration (8b, circles), which can be confirmed to be enlarged rough ER by electron microscopy (8c). Dissolution of the nuclear chromatin (8b, circles, 8c, N) indicates severe degeneration. This degenerating cell was identified to be β cell (8c, β), because of the presence of amyloid microfibrils (8c, Af) and membrane-bound insulin granules (figure 9, G). The damaged mitochondria are invested with rough ER membrane, forming autophagosomes (8d, Ap). a, b, H-E staining; c, d, Uranyl and lead staining.

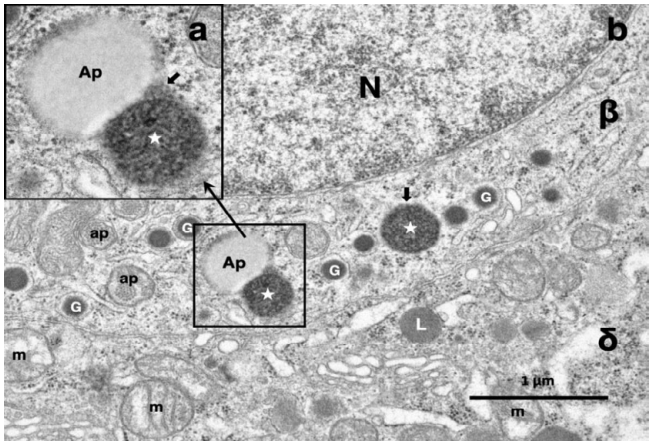

Figure 9: Degenerating β cell (9b, β) of the Langerhans islet, being characterized by membrane-bound insulin Granules (G), contains an autophagosome (Ap) in the process of binding with a lysosome (star) which shows evidence of membrane permeabilization (9 a,b, arrows). Please, note the marked difference with the normal Lysosome (L) of the neighbouring δ cell (δ). The degenerating β cell contains other autophagosomes (Ap) prior to the lysosomal digestion. N: Nucleus, m: Degenerating mitochondria of δ cell. Uranyl and lead staining. Figure 9a is a magnification of the square in figure 9b.

after Hydroxynonenal injections showed grossly similar ultrastructural features with the diabetic baboons in which severe β cell death and mild δ cell death were observed with the preceding amyloid depositions [18]. The differences between these two non-human primate models were that the monkeys showed necrosis of β cells, whereas δ cells showed merely degeneration with many autophagosome formations and mitochondrial degeneration (Figure 9b, m). In addition, the degenerating β cell of monkeys showed accumulation of many autophagosomes (Figure 8d, Ap; Figure 9, AP, ap). The lysosome in the process of binding with the autophagosome showed evidence of lysosomal permeabilization (Figure 9 a, b, stars, arrows).

Discussion

In summary, after Hydroxynonenal injections, neurons, hepatocytes and β cells similarly showed microcystic degenerations, dissolution of cell organella and the nuclear chromatin and scattered cell death with evidence of lysosomal membrane permeabilization and/or rupture. Although the similar findings were confirmed in the mice after Hydroxynonenal injections (data not shown), here the author focused the data obtained from monkeys. Hydroxynonenal has been increasingly recognized as a particularly important mediator of cellular dysfunction and degeneration in diverse disorders including Alzheimer's disease [23,24], cardiovascular disease, stroke, arthritis and asthma [25-28]. There is evidence that Hydroxynonenal also contributes to the dysfunction and death of pancreatic β cells in diabetes and alcohol-induced pancreatic damage [29,30]. The present results were completely in line with these reports and clearly demonstrated that Hydroxynonenal is toxic to diverse cells. It is suggested from these data that there should be a common mechanism in the cell death of different organs. Accordingly, the most important and largest problem to be addressed here is by which mechanism Hydroxynonenal can induce diverse cell death, *in vivo*.

Mechanism of lysosomal cell death

Epidemiological studies have shown that age is the chief risk factor for lifestyle diseases, but the molecular mechanisms that underlie their progression with aging remain unclear [31]. Lysosomes are membrane-bound organelles that mediate the intracellular degradation of macromolecules, so maintaining the lysosomal integrity and function is crucial for the cellular homeostasis. Recently, molecules that influence integrity of lysosomal membrane are thought to be closely related to cell survival and death as well as health and diseases. Diverse cell stresses with ageing can induce lysosomal membrane permeabilization/rupture, which ultimately leads to the programmed cell death by the spillage of cathepsins into the cytoplasm.

Since reactive oxygen species can induce the impairment of ER membrane integrity with the resultant release of Ca^{2+} into the cytoplasm, lipid peroxidation reactive product 'Hydroxynonenal' can be a trigger for the intracellular Ca^{2+} mobilization and subsequent calpain activation [32]. Concerning the molecular mechanism of ischemic neuronal death, Yamashima et al. formulated the 'calpain-cathepsin hypothesis' in 1998 [11]. Thereafter, concerning the mechanism of not only ischemic but also degenerative neuronal death, they suggested that Hsp70.1 (human type of Hsp70, also called Hsp72) with dual functions of molecular chaperone and lysosomal stabilizer becomes vulnerable to the cleavage by activated μ-calpain, which is facilitated after carbonylation by Hydroxynonenal [8,9,33]. Due to the Hydroxynonenal-induced carbonylation with the subsequent cleavage by activated μ-calpain, functional Hsp70.1 decreases steadily and this results in both accumulation of autophagosomes and permeabilization/rupture of the lysosomal membrane. Within the degenerating cell, the former appears as microcysts containing cell garbage and debris, while the latter causes lysosomal neuronal death (not apoptosis but necrosis). Interestingly, Shearn et al. confirmed both a decreased expression of Hsp70 in NASH by Western blotting and an increased carbonylation of Hsp70 by LC-MS/MS analysis of carbonylated proteins, compared to the normal human liver [34]. This was exactly consistent with the ischemic monkeys which showed delayed hippocampal neuronal death due to the carbonylation of the key site Arg469 of Hsp70.1 and the subsequent cleavage [8,9,12,13,33]. Calpain-mediated cleavage of carbonylated Hsp70 is closely related with lysosomal membrane

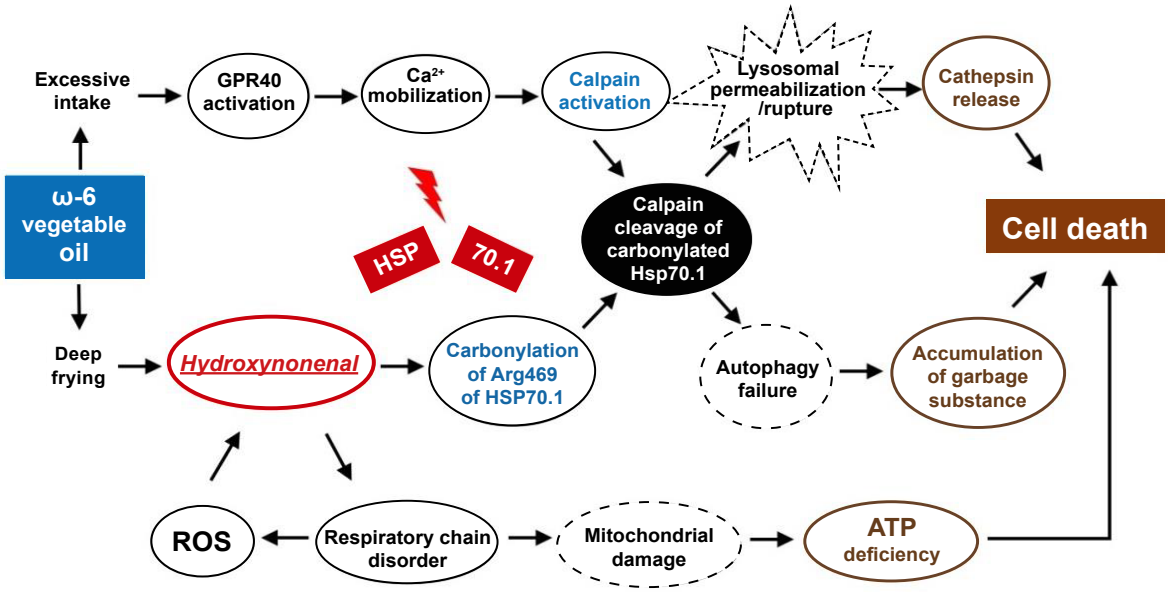

Figure 10: The calpain-cathepsin cascade explaining the role of ω-6-rich vegetable oil-derived 'Hydroxynonenal' in the cell degeneration/death.

permeabilization also in chronic liver diseases, because it represents the release of lysosomal protease cathepsins into the cytoplasm, then triggering hepatocyte cell death. Accordingly, it is tempting to speculate from these data that the same calpain-cathepsin cascade might be working for the cell death of neurons, hepatocytes and β cells by means of Hsp70.1 disorder (Figure 10). The similar molecular cascade was introduced in ischemic and Alzheimer neuronal death [8,9,11,13]. Hydroxynonenal induces not only mitochondrial damage and ATP deficiency but also autophagy/lysosomal failure with the resultant cell death. Excessive intake of ω-6-rich vegetable oil conceivably contributes to both GPR40 activation leading to calpain activation and Hydroxynonenal generation leading to Hsp70.1 carbonylation. These, combined together, would induce activated calpain-mediated cleavage of carbonylated Hsp70.1 with the resultant lysosomal permeabilization/rupture as shown in Figures 3a 3b and 7d.

Excessive fatty acids can cause cell death *via* GPR40

In the past two decades since the 'calpain-cathepsin hypothesis' was formulated, lysosomal cell death has been widely accepted to occur *in vivo* not only under pathological circumstances but also under physiological conditions [35-40]. An exquisite physiological example of non-apoptotic, lysosome-mediated programmed cell death is the post-lactational regression (involution) of the mammary gland. This is to remove alveolar mammary epithelium and return the gland to its pre-pregnant state, which is one of the complex and highly-regulated cell death programs occurring in the adult mammalian organism [36-38]. Milk fat globules during mammary gland involution upon cessation of lactation are known to be toxic to epithelial cells, since perturbation of lysosomal vesicle membranes by high levels of free fatty acids derived from milk triglycerides, results in the controlled leakage of cathepsins culminating in physiological cell death [38]. Feldstein et al. have previously demonstrated that incubation of liver cells with high concentrations (0.5 or 1 mM) of free fatty acids results in lysosomal membrane permeabilization and release of cathepsin B [39,40]. Furthermore, they showed that the lysosomal permeabilization process is preceded by lysosome translocation of Bax to cytosol and speculated that this proapoptotic member of the Bcl-2 family induces channel formation in the lysosomal membranes. Currently, the increment in dietary energy availability and sedentary lifestyle leads to obesity which causes an excessive accumulation of triglycerides in the liver and an elevated level of free fatty acids in the blood. Accordingly, it is likely that excessive free fatty acids are capable of inducing similar lysosomal cell death in hepatocytes as seen in alveolar mammary epithelium.

Here, the question to be addressed is why and how excessive amount of free fatty acids leads to lysosomal permeabilization/rupture. Given enlarged stores of triglycerides are probably harmless to the cells, free fatty acids should be directly cytotoxic, causing cell dysfunction and apoptotic cell death [41-44]. However, the signaling pathways triggered by free fatty acids still remain unclear. Intriguingly, overactivation of free fatty acid receptor GPR40 was found to induce disruption of Ca^{2+} homeostasis and cell death in response to excessive fatty acids in the high-fat diet [17]. In addition, conjugated linoleic acids and trans-arachidonic acids are known to affect body fat distribution, induce insulin resistance and stimulate insulin secretion also through GPR40 [45]. On the contrary, mice lacking GPR40 are protected from conjugated linoleic acid-induced fatty liver [46]. These data collectively suggest that GPR40 is possibly related to abnormal Ca^{2+} mobilization in response to excessive fatty acids, which presumably leads to the subsequent calpain activation with the resultant Hsp70.1 disorder and lysosomal permeabilization/rupture (Figure 10).

The most common source of Hydroxynonenal is an exogenous one, because it can be generated during food processing especially deep-frying of ω-6 PUFA-rich vegetable oil. Another common source is an endogenous one, because the reactive oxygen species produced by the mitochondrial respiratory (electron transport) chain trigger oxidation of membrane lipids. Furthermore, Hydroxynonenal can be produced by peroxidation of plasma Low-Density Lipoproteins (LDL) in the human body [47]. Although Hydroxynonenal is physiologically metabolized, under the severe and consecutive oxidative stresses, the intrinsic cellular detoxifying responses become overwhelmed and lipid peroxidation products accumulate to induce cellular injury [48,49].

Conclusion

In conclusion, ω-6 PUFA-rich vegetable oil-derived 'Hydroxynonenal' being capable of causing diverse cell degeneration/death, may be one of the important causative factors of lifestyle diseases such as Alzheimer's disease, NAFLD and NASH as well as type 2 diabetes. It is likely that appropriate amount of ω-3 PUFAs increase the serum BDNF level through the physiological GPR40 signaling pathway and contributes to the synaptogenesis and brain development [50]. In contrast, excessive amount of ω-6 PUFAs and the resultant Hydroxynonenal may cause cell degeneration *via* GPR40 overactivation and abnormal Ca^{2+} mobilization. By focusing dual aspects of the GPR40 signalling, further detailed studies are necessary to clarify the precise molecular cascade by which Hydroxynonenal cause diverse cell death [45]. Although it is still a long "distance" from the observed changes to the onset of Alzheimer's disease or type 2 diabetes, the present findings would bring some deep thinking about the molecular mechanism of Alzheimer's disease and related lifestyle diseases.

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