Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : April 18, 2024
Accepted : June 14, 2024
Published online : June 30, 2024
doi:10.24659/gsr.11.2_79

Original article

From fatty liver to steatohepatitis: Involvement of aldehydes

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Abstract

Over the past 80 years, we have been exposed to increased caloric intake associated with higher fat intake, concurrently with less physical activity. These lifestyle changes result in a marked increase in diseases associated with glycative stress, the condition characterized by excess aldehyde generation, such as obesity, type 2 diabetes, dyslipidemia, metabolic syndrome, and fatty liver/steatohepatitis. We describe our hypothesis as to how aldehydes are involved in the process of progression from fatty liver to steatohepatitis. Glycative stress is defined as an excess of aldehydes, and oxidative stress as an excess of free radicals and ROS (reactive oxygen species). In the body, carbohydrate- and fatty acid-derived aldehydes, which are produced in excess due to hyperglycemia and a high-fat diet, respectively, and modify proteins, lipids, and DNA by glycation. In our protection system, these aldehydes are, consuming NAD+ (nicotinamide adenine dinucleotide) and GSH (glutathione), catalyzed by GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ALDH (aldehyde dehydrogenase), and GLO (glyoxalase). Concurrently, oxidative stress is intensified by a decrease in GSH, and fatty acid-derived aldehydes are mostly produced by the oxidation of fatty acids. Therefore, as fatty liver progresses, the production of fatty acid-derived aldehydes increases significantly. An increase in the NADH/NAD+ ratio in hepatocytes leads to a decrease in lipid β-oxidation and the progression of fat accumulation. DNA modification modifies the promoter region of the low-density lipoprotein receptor related protein 1 (LRP1) gene, and decreased expression of LRP1 leads to abnormalities in lipid metabolism in hepatocyte. Once AGEs, products of protein glycation, stimulate RAGE in macrophages and Kupffer cells to increase the secretion of inflammatory cytokines, inducing inflammation. Glycation of procollagen stimulates Ito cells (hepatic stellate cells), and decreased GSH stimulates fibroblasts to increase collagen production, thus inducing fibrosis. When NAD+ is insufficient, the TCA cycle malfunctions and fumaric acid increases, which results in GAPDH being modified by succinylation and converted to S-(2-succinyl) cysteine-GAPDH, reducing activity and increasing unprocessed aldehydes, creating a vicious cycle. From the above, we deduce that the turning point from fatty liver to steatohepatitis is NAD+ deficiency caused by excess aldehydes, which in turn causes damage to the TCA cycle. We believe that measures against glycative stress (excess aldehydes) are important for preventing steatohepatitis. The involvement of LRP1 remains to be determined.

***KEY WORDS*:** glyceraldehyde, methylglyoxal, fumaric acid, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldehyde dehydrogenase (ALDH), glyoxalase, nicotinamide adenine dinucleotide (NAD+), glutathione

Introduction

We, humans, have faced struggles with oxidative stress in the process of evolution for several hundred thousand years, establishing defense systems against oxidative stress. This system is particularly elaborate in comparison with other animals. In contrast, it was only several decades ago when we started to fight glycative stress. Our society has been changing and we have problems with decreased physical activity due to developments in traffic systems and household electronic appliances, as well as excessive eating, especially of high fat foods. This led to remarkably increased

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glycative-stress, which is deeply related to diseases, such as obesity, metabolic syndrome, type 2 diabetes and hyperlipemia. This problem is a worldwide trend. Fatty liver, the topic of the present study, increases not only in morbidity and but also in severity. Cases of steatohepatitis are observed as the result of the combination in inflammation and fibrosis. Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) develop into cirrhosis with the rate of 10 % to 29 % within 10 years and 4 % to 27 % of patients have hepatic cancer [1, 2]. Risk factors of fatty liver (obesity, hyperlipemia, hyperglycemia and smoking) have a possibility of excessively generated aldehyde involvement. We examine what occurs in the development process from fatty liver diseases to steatohepatitis and infer its mechanisms from the perspectives of glycative stress. Medical treatments for steatohepatitis are challenging and complicated compared with simple fatty liver. The present attempts would be a significant step toward the establishment of treatments for steatohepatitis

1. Differences between oxidative stress and glycative stress

To begin, we reexamine oxidative stress and glycative stress from the viewpoints of field and classification. When oxidation-reduction reaction is simply defined as electron exchange, the boundary between the two types of stress is confusing. Oxidative stress and glycative stress can be accurately distinguished with simple definitions to be understood. Glycative stress refers to the state of excessive aldehyde formations in the body. Aldehydes are highly reactive and react with serum proteins in blood and membranes of vascular endothelial cells. Aldehydes are constantly produced and eliminated, and are detected in the blood in equilibrium. Furthermore, aldehydes easily react with protein and lipids in cells through cell membranes. Moreover, they react with cytosine (DNA constituent base) through nuclear membranes. Products of aldehyde-protein reactions are designated as advanced glycation endproducts (AGEs) including protein carbonyl [3, 4]. Amadori-glycated phosphatidylethanolamine (Amadori-PE) is a glycation product of lipids [5].

Oxidative stress refers to the state of excessive free radical and reactive oxygen species (ROS). Antioxidants such as Vitamin C and E reduce oxidation reactions. These antioxidants, however, do not suppress glycation reaction where aldehydes and biomolecules bond.

The leading role is played by aldehydes in glycative stress reactions. Aldehydes are mainly classified into carbohydrate-derived and lipid-derived aldehydes based on formation paths (***Fig. 1***) [6, 7]. The former is a short chain aldehyde due to "simultaneous formations of diversified aldehyde in a chain reaction manner," which follow blood glucose spikes as postprandial high blood glucose [8]. We designate this phenomenon as an "aldehyde spark" [4]. Parts of reducing sugars (*i.e.*, glucose, fructose) become a ring-opening aldehyde, where aldehyde groups are exposed. Consequently, they react with substances around them and induce injury to vascular endothelial cells. Ring-opening glucose (the number of carbons: 6) are stimulated by other aldehydes, oxidative stress, heat and ultraviolet radiation, which induce further formations of short-chain aldehydes (the number of carbons: 2 and 3). Among them, AGEs, which are produced from glyceric aldehyde (GA), are the most toxic. This is designated as toxic AGE (TAGE) [9, 10]. The latter, lipid-derived aldehydes, are produced via oxidation of fatty acid. Among them, methylglyoxal (MGO), which is an aldehyde produced from carbohydrates and fatty acids, is related to the onset of multiple diseases in the body.

During the demethylation process of epigenetic changes, formaldehyde is produced from the released methyl groups [11-14]. Therefore, formaldehyde levels of 0.05 ~ 0.1 mM are physiologically detected in the blood of healthy individuals. It is said that organic mercury (methylated mercury), the cause of Minamata disease, is demethylated in the liver to produce formaldehyde [15]. Naturally, both DNA and histone proteins are modified by aldehydes.

As measuring fatty-acid-derived short-chain aldehydes in blood had been difficult, it has been difficult to attract attention to this aldehyde [6]. However, fatty-acid-derived aldehydes are deeply related to the onset and aggravation of diseases in brain tissues and bones, particularly bone marrow where lipids are abundant. Fatty-acid-derived aldehydes are produced in the body with oxidative stress condition and exert diverse biological effects. It has been regarded that 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) are causative factors of alcoholic hepatopathy [16, 17]. Normal hepatic parenchymal cells have a small distribution as fat. Fatty liver, however, has a risk of increasing fatty-acid-derived aldehydes. Experiments with NASH model mice, where eicosapentaenoic acid was administered (EPA) to the control group, an omega-3 unsaturated fatty acid suggested the depletion of glutathione in liver tissues and the exacerbation of fibrosis in the liver [18]. This phenomenon is caused by the excessive formation of fatty-acid-derived aldehydes. It was suggested that a large amount of GSH, a cofactor, was consumed due to the metabolization of glyoxalase (GLO) [19].

The research of glycative stress started with the examinations of Maillard reaction in food. The initial reaction in glycation is a carbonylation reaction, where an exposed aldehyde group in a ring-opening glucose (-CHO) and an amino group of protein/peptide ($-NH_2$) are reacted, and consequently, Schiff base is formed (a chemical formula is supposed but this compound does not exist as is transiently formed). The present study designates reactions of aldehyde groups and amino groups as carbonylation and all the sequential reaction as glycative reaction.

Short-chain fatty acids (acetic acid, butyric acid and propionic acid), which are produced by mutualistic bacteria of intestinal flora, contribute to the prevention and progression of fatty liver [20-23]. Short-chain fatty acids have an inhibitory activity for the AGEs formation *in vitro* (the inhibition of protein carbonylation) [23] and a promotive activity for base metabolism via specific receptors *in vitro* [24]. However, oral administrations of short-chain fatty acids have no effects on receptors because they are subsequently digested and absorbed [25].

Another type of aldehyde is derived from food and drinking items for pleasure without the purpose of nutrition. One is alcohol-derived aldehyde. The other is cigarette smoking-derived aldehyde (***Table. 1***). General perception shows that smoking is related to oxidative stress. However,

the smoke of cigarettes contains multiple aldehydes. The skin of smokers has a high level of skin autofluorescence values (*Fig. 2*)[26]. Mortality rate is twice as high in smokers than non-smokers in the USA. Similarly, smoking increases the mortality rate of fatty liver twice as much[27]. Moreover, liver filament formations are remarkably aggravated by smoking and Fibro Scan® shows progression of liver sclerosis[28], which elevates incidence rates of malignant tumors[29]. Therefore, people with drinking and/or smoking habits have more intense glycative stress.

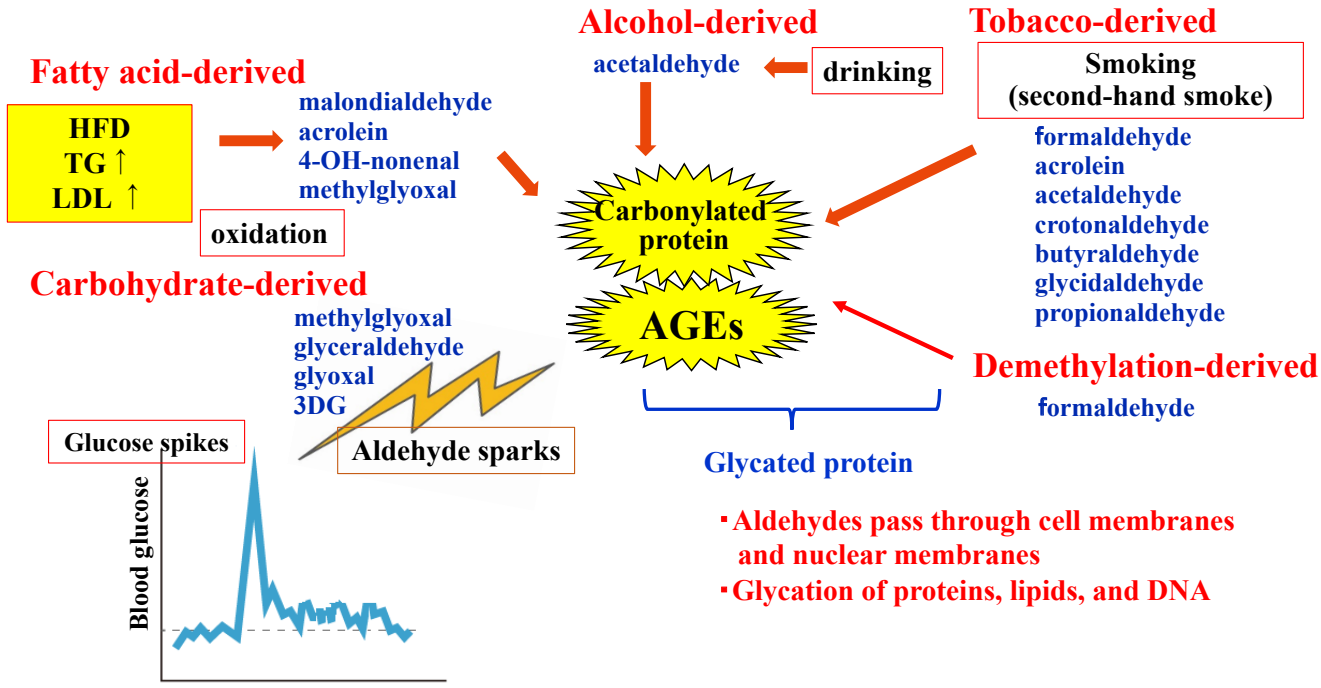

Fig. 1. **The main characters are aldehyde. Where do they come from?**

Aldehydes produced in the body are mainly derived from carbohydrates and fatty acids. Trace amounts of formaldehyde are produced with the demethylation of DNA and histone proteins. Exogenous aldehydes come from drinking alcohol and smoking. HFD, high fat diet; TG, triglyceride; LDL, low-density lipoprotein; 3DG, 3-deoxyglucosone; AGEs, advanced glycation endproducts,

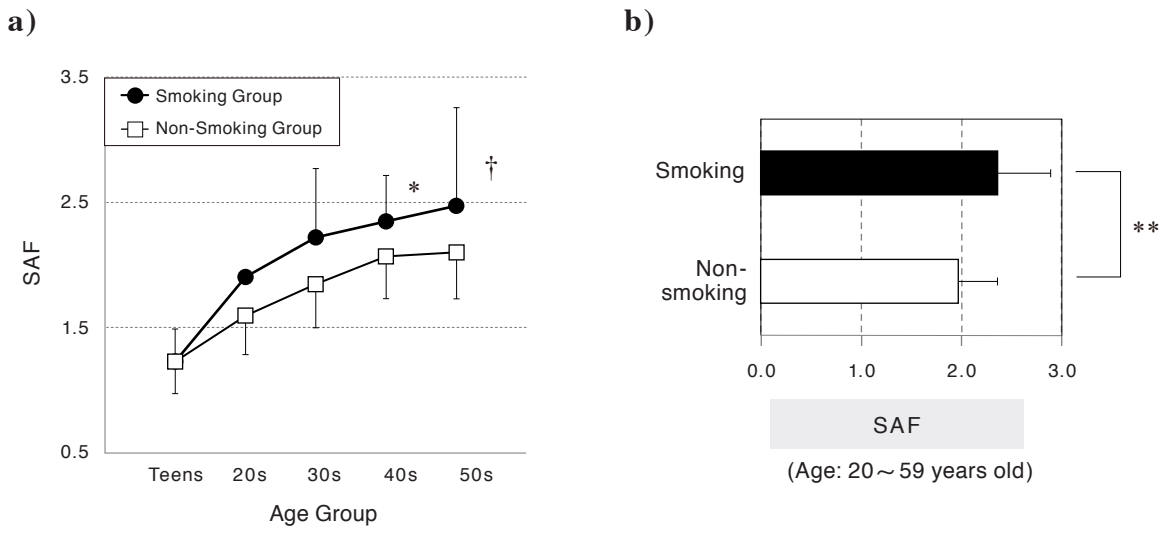

Fig. 2. **Aging trends in SAF and the impacts of smoking.**

The results are expressed as mean ± standard deviation, † $p < 0.1$, * $p < 0.05$, ** $p < 0.01$m Mann-Whitney's U-test. **a)** n = 244, **b)** Subject's ages between 10 to 59. Smoking group, n = 33; Non-smoking group, n = 177. SAF, skin autofluorescence measured by AGE Reader (DiagnOptics, The Netherlands). The figures are created based on reference 26).

Table 1. Toxic substances contained in cigarette smoke.

acetaldehyde	chromium	lead	N-nitrosomethylethylamine
acetamide	chrysene	MeA-a-C (2-amino-3-methyl-9H- pyridoindole)	N-nitrosomorpholine (NMOR)
acetone	cobalt	Mercury	N-nitrosonornicotine (NNN)
acrolein	coumarin	methyl ethyl ketone	N-nitrosopiperidine (NPIP)
acrylamide	cresol	5-methyl chrysene	N-nitrosopyrrolidine (NPYR)
acrylonitrile	croton aldehyde	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	N-nitrososarcosine (NSAR)
aflatoxin B	cyclopentapyrene	naphthalene	nornicotine
3-aminobiphenyl	dibenzanthracene	nickel	phenol
ammonia	dibenzopyrene	nicotine	PhIP (2-amino-1-methyl-6-phenylimidazo pyridine)
anabasine	2,6-dimethylaniline	nitric oxide (NOx)	polonium 210
o-anisidine	ethyl carbamate (urethane)	nitrobenzene	propionaldehyde
arsenicum	ethylbenzene	nitrogen monoxid	propylene oxide
A-α-C	ethylene oxide	nitromethane	pyridine
benzaldehyde	formaldehyde	2-nitropropa vinyl acetate	quinoline
benzanthracene	furan	N-nitrosoanatabine (NAB)	resorcinol
benzene	Glu-P-1 (2-amino-6-methyl pyrrolidone)	N-nitrosoanatabine (NAT)	selenium
benzofuran	Glu-P-2 (2-aminodipyrrolid)	N-nitrosodiethanolamine (NDELA)	styrene
benzopyrene	glycidaldehyde	N-nitrosodiethylamine 2	o-toluidine
beryllium	glycidol	N-nitrosodimethylamine (NDMA)	toluene
1,3-butadiene	hydrazine	N-nitrosomethylethylamine	Trp-P-1 (3-amino-1,4-dimethyl-5H- pyridoindole)
butylaldehyde	hydrogen cyanide	N-nitrosomorpholine (NMOR)	Trp-P-2 (1-methyl-3-amino-5H- pyridoindole)
cadmium	hydroquinone 3	N-nitrosonornicotine (NNN)	uranium 235
carbon monoxide	indenopyrene	N-nitrosopiperidine (NPIP)	uranium 238
catechol	IQ (2-amino-3-methylimidazole)	N-nitrosopyrrolidine (NPYR)	vinyl acetate
chlorinated dioxin / furan	isoprene	N-nitrososarcosine (NSAR)	vinyl chloride

Red letters indicate aldehydes. Not only carcinogens and oxidation-inducing substances, but also diverse aldehydes are contained.

2. *Liver as a defense system against glycative stress*

The human body has anti-glycation systems. (***Fig. 3***) Among them, the liver plays the vital role. In hepatocyte, defense systems for aldehydes exist abundantly, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glyoxalase, (GLO), and aldehyde dehydrogenase (ALDH) (***Fig. 4***).

• GAPDH

GAPDH accounts for approximately 20% for protein in hepatocyte and is the most highly distributed enzyme in the body. GADPH plays the key role in glycative stress defense[30,31]. This exists as a tetramer in cells and serves as a glycolytic enzyme. GAPDH is related to the GA metabolism. Glyceraldehyde (GA), which is moved into or formed in hepatocyte, is intensively phosphorylated by triokinase[32,33],

Enzymes	Materials	Organs/Cells/Organelles
【Aldehyde metabolism】 GAPDH ALDH Glyoxalase I &II 【AGE breaking】 OPH	【Aldehyde trapping】 amino acids 【Soluble RAGE】 【AGE generation control】 short chain fatty acids 【AGE breaking】 melatonin	【Phagocytosis】 fibroblasts・glial cells reticuloendothelial cells macrophage・monocyte 【Organelles】 RAGE scavenger receptors for AGEs lysosome・proteasome 【Excretion organs】 liver (hepatocyte) kidney (podocyte)

Fig. 3. **Anti-glycation system in human.**

Glycation products are processed through various cells/intracellular organelles, metabolized and subsequently eliminated by the liver and kidneys. Hepatocytes are rich in aldehyde metabolizing enzymes, *i.e.*, GAPDH, ALDH, and GLO, while the AGEs metabolizing enzyme OPH is expressed in skin and various tissues in addition to the liver. Various materials are involved in the anti-glycation system. Representative endogenous substances are shown in parentheses. Amino acids trap aldehyde, short-chain fatty acids derived from intestinal bacteria inhibit AGEs formation, and melatonin promotes AGE cleaving as well as its antioxidant effect. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALDH, Aldehyde dehydrogenase; GLO, glyoxalase; OPH, oxidized protein hydrolase; AGEs, advanced glycation endproducts; RAGE, receptor for AGEs.

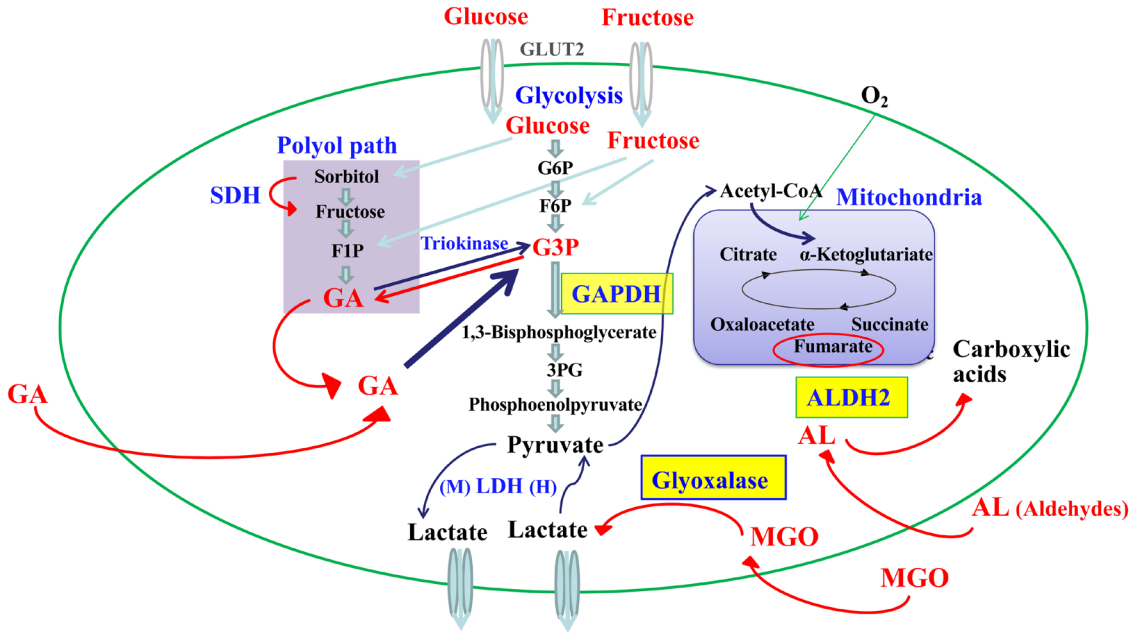

Fig. 4. **Glucose metabolic pathway and involvement of aldehydes in hepatocytes.**

In hepatocytes, aldehyde excess activates GAPDH, ALDH2, and glyoalase, while glucose excess activates the polyol pathway. When aldehydes enter the cell, they are promptly phosphorylated and retained inside the cell to be metabolized by enzymes. Much NAD+ and GSH are consumed as compensation for enzymatic activity. The influence extends to mitochondrial function. GLUT2, glucose transporter 2; AL, aldehydes; GA, glyceraldehyde; MGO, methylglyoxal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALDH, aldehyde dehydrogenase, SDH, sorbitol dehydrogenase; LDH, lactate dehydrogenase; G6P, glucose-6-phosphate; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate.

and converted into GAP, which is membrane-impermeable. GAP is metabolized by GAPDH with the assistance of NAD^+ and is converted into 3-phosphoglycerate (3PG) (*Fig. 5*). It seems like there is a mission in the hepatocyte to metabolize all GA and protect our body from GA. Although GAPDH is located in the glycolytic pathway, it is not a rate determining enzyme because of its large amount. It is suggested that the protection system is ready for emergent risks of the mass production of GA.

GAPDH is a protein with moonlighting functions. GAPDH, which has a high affinity to DNA, has a function of DNA repair enzyme, as a monomer of uracil-DNA glycosylase[34,35]. When intensive glycative stress induces the excessive formation of intracellular aldehydes, abnormal proteins are increasingly produced from intracellular protein due to aldehyde modification and consequently, increased endoplasmic reticulum (ER) stress induces insufficient processing activities of proteasome. This would be "the state in the direction to cell death." In this condition, GAPDH changes its behavior from the role of guardian to the promoter of apoptosis[36]. GAPDH moves into mitochondria, and induces the acceleration of membrane permeability, the loss of transmembrane potential and the release of apoptosis inducer[37,38]. This is regarded as a trigger of apoptosis[30]. When GAPDH forms monomers and dimers via nuclear translocation, it serves to function as a protein promoting apoptosis[39-41]. GAPDH in cells binds with integral membrane proteins, endoplasmic reticulum Ca^{2+} pump. Consequently, Ca^{2+} intake of cytoplasm increases[42,43]. Finally, cells result in apoptosis.

- **Aldehyde dehydrogenase (ALDH)**

The ALDH super family, which has 19 types of isozymes, plays a significant role for sustaining biological functions[44,45]. Among them, ALDH2 is a detoxifying enzyme with a high affinity for acetaldehyde produced by alcohol drinking[46]. This enzyme expresses in the liver and the kidney as well as mitochondrial matrix in cells. ALDH degrades multiple types of aldehydes including acetaldehyde (*Fig. 5*)[47-50]. Glyoxal, methylglyoxal and others of aldehydes are also substrates of ALDH2[51]. It has been recognized that ALDH2 metabolizes fatty-acid-derived aldehydes such as 4HNE and MDA[52-54]. NAD+, a cofactor, is required for ALDH to exert enzyme reactions. Therefore, in the condition of intense glycative stress, that is, the state of excessive formation of aldehydes, a multitude of NAD+ are consumed.

Excessive aldehyde formation induces the shortage or depletion of NAD+ (nicotinamide adenine dinucleotide) in the process of metabolization by GAPDH and ALDH, as NAD+ is necessary for metabolization. Individuals who drink alcohol (excessive ethanol consumption) should be careful because NAD+ is also consumed when ethanol is converted to acetaldehyde by alcohol dehydrogenase.

When superfluous glucose increases in cells, the polyol pathway is activated. This is an alternative pathway for extra glucose which the glycolytic pathway does not manage to process, where extra glucose is converted into fructose not using a glycolytic pathway. Glucose is converted into sorbitol via aldose reductase and subsequently, sorbitol is converted into fructose via sorbitol dehydrogenase (SDH). However, SDH catalyzes reactions by consuming NAD+. This assists leading to shortage or depletion of NAD+. Some parts of fructose are converted into GA and the amount of GA increases. This leads to an increase in aldehyde generation. Both events cause vicious cycles.

TCA cycle of mitochondria requires NAD+ at three points through reactions. Insufficient NAD+ hinders a steady cycle and reduces the formation of ATP (*Fig. 6*).

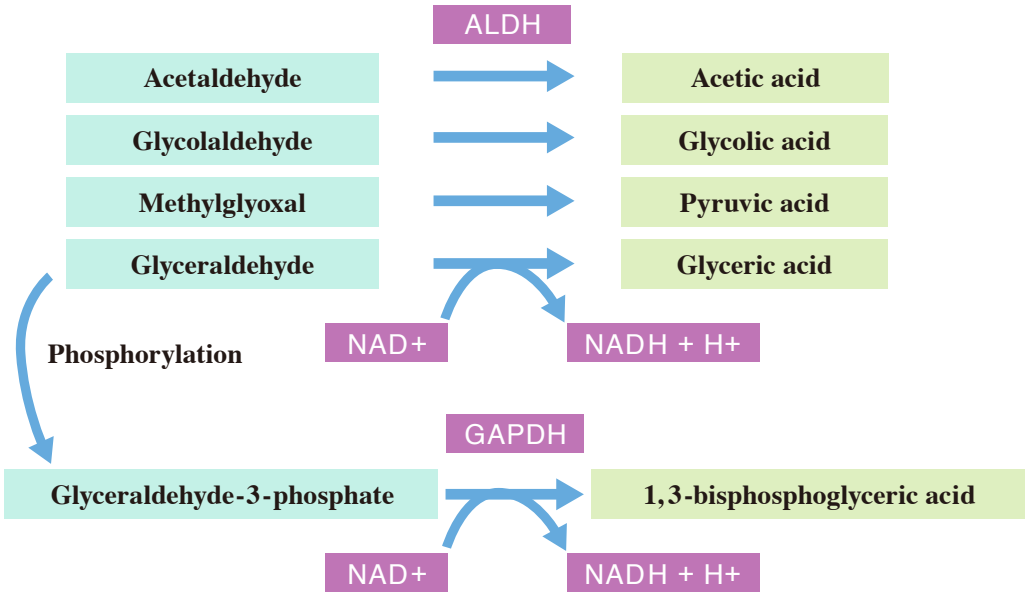

Fig. 5. **Aldehyde metabolizing enzyme that utilizes the cofactor NAD+.**

NAD+ is consumed as a cofactor, glycative stress with aldehyde excess condition leads to NAD+ deficiency. NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form).

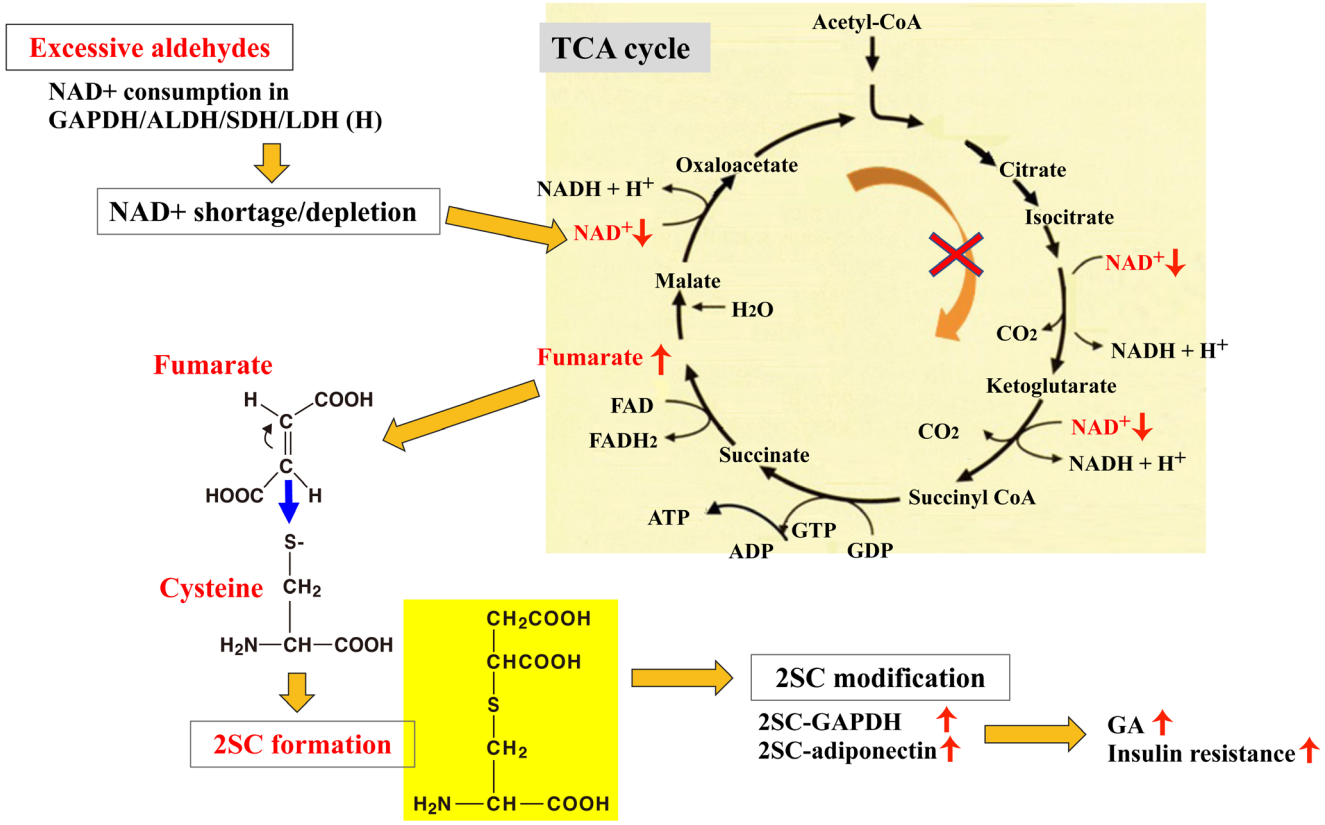

Fig. 6. **TCA cycle.**

Under the condition with NAD+ deficiency, the circuit does not run smoothly and ATP production decreases. Subsequently, fumaric acid increases. When fumaric acid binds to GAPDH (succinylation), its activity is attenuated. AcetylCoA. acetyl coenzyme A; NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); FAD+, flavin adenine dinucleotideadenine dinucleotide; FADH2, flavin adenine dinucleotideadenine dinucleotide (reduced form); ATP, adenosine triphosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALDH, aldehyde dehydrogenase; SDH, sorbitol dehydrogenase; LDH (H), lactate dehydrogenase (H type); 2(SC), S-(2-succinyl)cysteine; GA, glyceraldehyde.

Dysfunctions in the TCA cycle reduce the formation of NADH (nicotinamide adenine dinucleotide, reduced form) and $FADH_2$ (flavin adenine dinucleotideadenine dinucleotide, reduced form), which results in the decrease of supply to the electron transfer systems. Therefore, the formation of ATP decreases in the electron transfer system. As a result, a shortage of energy production is caused in mitochondria and diverse functions decrease in hepatocyte.

- **Glyoxalase (GLO)**

There are two types of GLO, glyoxalase I (GLO1) and glyoxalase II (GLO2), where the GLO system is composed [55]. They express in multiple cells such as the liver and the kidney. Methylglyoxal (MGO) is converted into Sd-lactoylglutathione by GLO1, which is bound by glutathione (GSH) through a catalytic role like a cofactor. Furthermore, it is hydrolyzed to D-lactic acid via GLO2. Finally, D-lactic acid is metabolized to pyruvic acid. As MGO is formed both in carbohydrates and fatty-acid-derived manners, MGO is associated with diverse glycative-stress-related diseases in the onset and progression. Therefore, this enzyme which metabolizes MGO plays a significant role in defense functions for glycative stress.

The increased MGO production and the excessive burden on GLO lead to the state of deficient GSH. This finding is frequently observed in progressed fatty livers and steatohepatitis. On the contrary, the depletion of GSH induces severe liver disorders. For example, acetaminophen (APAP) overdose induces a liver dysfunction [56] caused by the depletion of GSH which inhibits the activities of GLO, and consequently, MGO markedly increases.

- **Oxidized protein hydrolase (OPH)**

OPH is a type of serine protease and is the same as acylpeptide hydrolase (ACPH) with free protein N-terminal acylation amino acid [57]. Furthermore, it is designated as an acylamino-acid-releasing enzyme (AARE) [58]. OPH, which exists in diversified tissues such as human red blood cells, livers, kidneys, brains and hearts, degrades above-mentioned oxidized proteins and glycated proteins on a preferential basis [59].

3. Epigenetic changes and glycative stress

Epigenetic changes refer to a chemical modification of DNA with a purpose of turning gene control on/off. In a case of controlled physiological modification, a base sequence does not alter and is remembered as cell characteristics. The base sequence is continued as genetic inherence. Physiological DNA methylation, via methyltransferase (DNA methyltransferase), has the addition of a carbon to the methyl group, the fifth position of cytosine base with CpG sequence. Consequently, it is converted into 5-methylcytosine. As a result, gene expressions are affected and the inhibition of expression could occur. Via demethylating enzymes, the methyl group is released and it returns to cytosine. Then, gene expression returns to the original state.

Methylation age, a measurement system of biological age, is an attempt where aging degrees of cells are assessed based on assessments of methylation degrees of DNA. Overview of the measurement method is as follows: first, the bisulfite processing is conducted. Unmethylated cytosine in DNA is converted to uracil[60,61]. Contrarily, cytosine which is not methylated and not modified in other manners remains as cytosine without any conversions. Therefore, after bisulfite processing, types of DNA with different base sequences are expressed. When analyzing differences with the use of a DNA sequencer, where and how frequently methylations have occurred were detected. However, this method of methylation age does not provide accurate examinations of pure methylated cytosine. Other than methylation, multiple modifications are included, such as dimethylation, trimethylation, carbonylation and alkylation.

Methylation age measurements have provided multiple findings. Along with aging, methylation age is aging. It has been suggested that methylation age is accelerated in the state of intensive glycative stress, including the presence of diabetes[62,63]. It is notable that repeated spikes of glucose and hyperlipidemia, inducing excessive aldehyde generation, promote DNA methylation[64]. The reason is that the presence of excessive aldehydes induces cytosine carbonylation in a non-physiological aspect. Glycative stress definitely affects epigenetic changes.

4. Process from fatty liver to steatohepatitis

Healthy livers, which have glycogen granules and lipid droplets in the cytoplasm, play the role of energy storage. Lipid droplets are organelles, which exist in not only the liver and adipose cells but also cells throughout the body. Lipid droplets store lipids and toxic lipids, having functions to protect the body from intracellular lipotoxicity[65]. Healthy persons have a limited amount of lipid droplets in cytoplasm. However, lipid droplets are observed in intranuclear areas of patients with steatohepatitis. Its causative factors are unidentified. We speculate that lipid droplets are rich in Amadori-PE in the steatohepatitis.

Simple fatty liver refers to the state of stored (macrovesicular) lipid droplets in hepatocyte. Lipid droplets consist of a phospholipid monolayer (membrane of endoplasmic reticulum) wrapping nucleus of triacylglycerol and cholesterol ester[66]. Simple fatty livers carry a benign prognosis. Even in a case of considerable lipid storage, cirrhosis and liver cancer do not typically develop.

Steatohepatitis refers to a pathological condition, where cytopathy, necrosis, inflammation and fibrosis are added to fatty liver (***Fig. 7***). Alcohol drinking (ethanol ingestion) induces NAD+ consumption, due to ethanol/acetaldehyde metabolization via alcohol dehydrogenase and sequentially, ALDH. This leads to the formation of reduced form NADH. Excessive NADH stimulates fatty acid synthesis in hepatocyte. An excess of NADH (an increase in the NADH/NAD+ ratio) stimulates fatty acid synthesis in hepatocyte and reduces fatty acid β-oxidation, resulting in fat deposition and the progression of fatty liver[67]. NAD+ functions as a cofactor for multiple enzymatic reactions, many of which are significant reactions. Therefore, the disturbance of equilibrium caused by glycative stress (excessive aldehydes) could be a boundary to distinguish traveling directions between remaining as fatty livers and progressing to steatohepatitis. Steatohepatitis is developed when infiltration of inflammatory cells, mainly neutrophil, macrophages and Kupffer cells, as well as inflammatory changes are added to fatty liver in this condition. The progression of steatohepatitis leads to liver cirrhosis due to the aggravation of fibrosis. This may be a pathogenesis of cancers in the liver. At the time of diagnosis, the range of steatohepatitis is extensive from a case with only little fibrosis to hepatic cirrhosis. How fibrosis developments progress decides the resulting prognosis. There are cases of fatty liver/inflammatory liver cirrhosis where only a slight lipid droplet remains or lipid droplet disappears in hepatocyte.

- **Inflammation**

Neutrophil is dominant in inflammatory cells observed in steatohepatitis. In addition, macrophage and Kupffer cells are recognized. RAGE, which expresses in these cells, binds with AGEs and then, inflammatory cytokines (*i.e.*, TNFα [tumor necrosis factor α], IL6 [interleukin-6]) are secreted. Thus, inflammation is induced. Consequently, cellular injuries are induced.

It was experimentally suggested by Leung C et al. that glycative stress aggravates steatohepatitis[68,69]. The experiments, where rats were fed a methionine/choline deficient diet for six weeks and then, the methionine choline deficient (MCD) diet containing high AGEs for another six weeks, suggested that steatohepatitis progressed[68]. In comparison with an MCD diet alone, the MCD diet containing high AGEs increased the content of liver AGEs. This elevated the triglycerides level, the formation of NADPH-dependence-ROS, 4HNE addition, fatty degeneration, and inflammatory cytokines, and fibrosis index (α-SMA [α-smooth muscle actin], CTGF [connective tissue growth factor], COL1A and picrosirius). These findings suggest the possibility that inflammations are developed via the secretion of inflammatory cytokines when AGEs bind with RAGE on macrophage and Kupffer cells of liver tissues.

Study with an experiment suggested that steatohepatitis with fibrillization occurred after 33 weeks of feeding mice a high fat, high fructose and high cholesterol (HFHC) diet[69], induced an increase of fatty-acid-derived aldehyde, 4HNE, and increased gene expression of α-SMA and type 1 collagen as a fibrosis index. This model suggests that the formation of carbohydrate-derived aldehyde due to the excessive ingestion

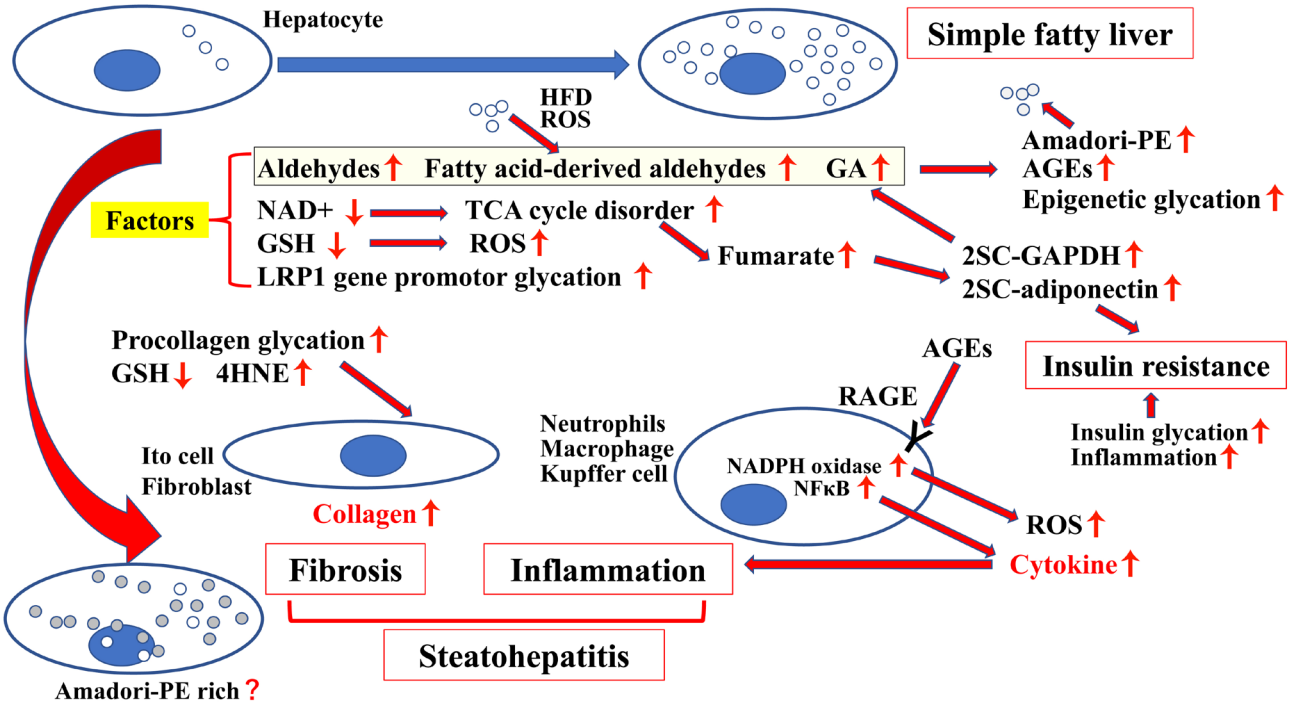

Fig. 7. **From fatty liver to steatohepatitis.**

Excess aldehydes cause proteins, lipids, and DNA to undergo glycation modifications. This places a strain on aldehyde metabolic enzymes, which consume and decrease NAD+ and GSH. Fibrosis progresses in fibroblasts and Ito cells. RAGE in immune response cells is stimulated, releasing inflammatory cytokines. Amadori-PE increases in lipid droplets. The combined effects of these changes lead to steatohepatitis. The involvement of LRP1 remains to be determined. HFD, high fat diet; ROS, reactive oxygen species; GA, glutaraldehyde; 4HNE, 4- hydroxynonenal; AGEs, advanced glycation endproducts; RAGE, receptor for AGEs; Amadori-PE, Amadori-glycated phosphatidylethanolamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD+, nicotinamide adenine dinucleotide; GSH, glutathione; LRP1, low-density lipoprotein receptor related protein 1; 2(SC), S-(2-succinyl)cysteine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NADH, nicotinamide adenine dinucleotide (reduced form); NFκB, nuclear factor κB; Ito cell, hepatic stellate cell.

of fructose antecedently occurs followed by chain reactions.

Enhanced ROS production via the RAGE/NADPH-oxidase signaling pathway, combined with the oxidation of increased fatty acids by a high-fat diet, further enhances the production of fatty acid-derived aldehydes (*i.e.*, MGO), forming a vicious cycle in the mechanism leading from fatty liver to steatohepatitis. The consumption of GSH during the metabolism of MGO by GLO increases oxidative stress, further adding to the vicious cycle.

This experiment concluded that the diet containing AGEs alone did not induce fatty liver symptoms or steatohepatitis in normal livers of animals which were normally fed [69].

- **Fibrosis**

It has been known that aldehydes promote liver fibrosis, which is a cause of alcoholic liver disease. Typical collagen formation in the liver is a fibroblast recognized as Ito cell, which is also designated as hepatic stellate cell. Ito cells store vitamin A and exist in the space of Disse, which a small area between the sinusoids and hepatocytes.

Ito cells are activated during liver injury and change to a myofibroblast-like morphology, resulting in increased collagen production. They are also closely involved in the pathogenesis of liver cirrhosis. AGEs promote the activation and proliferation of Ito cells, and said to be leading to the formation of ROS [68].

Intact procollagen inhibit the collagen synthesis via Ito cells in a concentration-dependent manner. However, when carboxyl-terminal telopeptides of procollagen are modified by acetaldehyde, Ito cells are not able to distinguish from normal procollagen. The collagen production increases due to negative-feedback-inhibition. In livers of patients with alcoholism, procollagen and procollagen-related substances are formed and fibrosis progresses. The content of GSH in Ito cells decreases, and consequently, the expression of collagen type 1 mRNA and the formation of collagen protein increases [70]. Other than acetaldehyde, fatty-acid-derived aldehydes, including 4-hydroxynonenal, stimulate the collagen formation in liver fibroblasts, which leads to the promotion of fibrosis [67].

- **Insulin resistance**

Steatohepatitis is invariably accompanied by insulin resistance. Among the factors that increase insulin resistance in IRI (immuno-reactive insulin) that we measure, the most important contributor is aldehyde excess. The reason is that aldehydes have an impact from the stage of insulin

biosynthesis in pancreatic β cells. β cells undergo a biosynthesis of insulin via pre-proinsulin and proinsulin (*Fig. 8*)[4].

When Arg 31-Arg 32, which connect insulin B-chain and C-chain, and Lys 64-Arg 65, which connect C peptide and A-chain, undertake glycative modifications, it is difficult to cleave by protease at C-chain terminal. Therefore, ratios of proinsulin/insulin in cells elevate. Proinsulin and insulin are secreted maintaining this ratio with responses to glucose stimuli. However, IRI, which we measure, includes both proinsulin and insulin. In patients with diabetes, 9% of insulin is modified with glycation[71]. Glycated insulin does not have insulin resistance. In the state of excessive aldehydes, the composition ratio of active intact insulin is notably decreased in IRI (*Fig. 8*). This is the reality of insulin resistance.

• Abnormality in mitochondrial functions

Hepatocyte plays the key role of defense system for excessive aldehydes. GAPDH, ALDH and GLO are forced to exert their full capacities consuming NAD+ and GSH. Insufficient GHS inefficiently eliminates ROS. Fatty acid which is contained in lipid droplets inside hepatocytes are oxidated at a rapid pace. This leads to a vicious cycle where further fatty-acid-derived aldehydes increase. Moreover, the shortage of NAD+ affects the mitochondrial TCA cycle, which hinders a smooth cycle. Consequently, in addition to the reduction of ATP production, the volume of fumaric acid increases (*Fig. 6*)[72-74].

Fumaric acid reacts with cysteine residue (-SH) of protein/peptide and S-(2-succinyl) cysteine (2SC) is formed. This is another type of post-translational modification of proteins. Fumaric acid, which is produced in mitochondria, reacts with GAPDH as well. This induces 2SC-GAPDH generation. As a result, the enzyme activities of GAPDH decrease[72]. This results in the state where metabolic capacities significantly decrease for GA, which is formed as carbohydrates-derived aldehyde, GA, which is formed in glycolysis, and GA, which is formed in polyol pathway.

It is observed that model mice created by feeding a high fat diet exhibit a reduction of protein level in liver tissue GAPDH as well as the elevation of GA and MGO in the plasma[7,75]. The mechanism is inferred as follows: GAPDH and ALDH exert full capacities to respond to the excessive fatty-acid-derived aldehydes via the induction of a high fat diet. The enzyme activities decrease due to the 2SC-GAPDH formation, which is induced by fumaric acid via the functional abnormality of the TCA cycle. Consequently, plasma GA increases. As the reactivity of GA is significantly high, GA readily reacts with monosaccharides and fatty acids. Consequently, diverse short-chain aldehydes are generated in a chain reaction manner. Plasma concentration of MGO, which is also generated from carbohydrate and fatty acid, is 300～400 nmol/L at fasting and increases by 30-40% after 30 minutes of oral ingestion of 75 g of glucose[8].

• Non-physiological epigenetic alteration of LRP1

Low-Density Lipoprotein Receptor-Related Protein 1

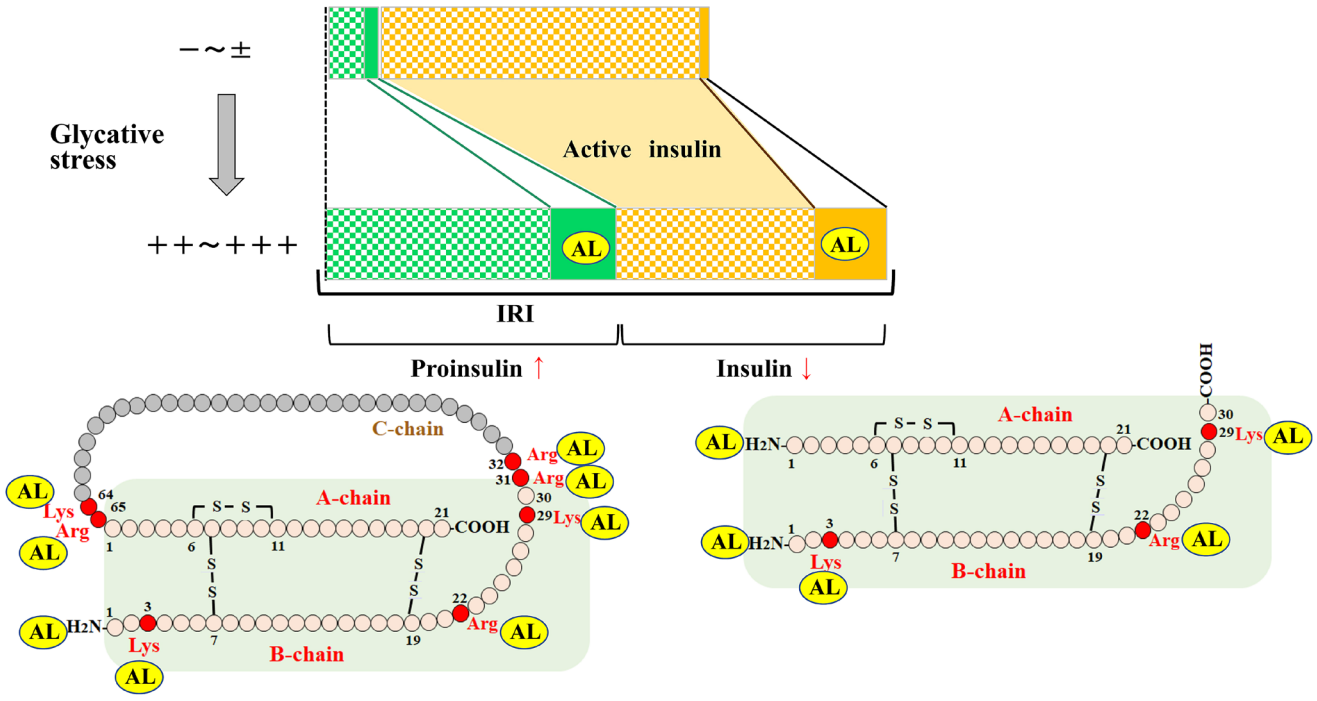

Fig. 8. **Glycative modification in the stage of insulin biosynthesis.**

The process of insulin biosynthesis in pancreatic β cells is affected by glycation stress (excess aldehydes). When the residues of Arg or Lys of the proinsulin B chain are glycated, it becomes protease resistant and the C chain cannot be cleaved. This increases the rate of proinsulin secretion and increases the IRI value, which cross-recognizes proinsulin and insulin. Glycated insulin has no insulin activity, so the rate of active insulin in the IRI decreases. AL, aldehyde; IRI. immuno-reactive insulin.

(LRP1) takes on a well-rounded role in plasma clearance of chylomicron and VLDL remnant lipoprotein. Its dysfunctions are related to obesity, diabetes and fatty liver diseases[76,77]. LRP1, which expresses abundantly in the liver, functions in multiple physiological processes; the removal of diverse circulatory molecules, such as proteinase inhibitor complex, serpin enzyme complex, chylomicron remnant and activated clotting factor. It is reported that LRP1 polymorphism is related to the elevation of triglycerides, the reduction of HDL level[78,79], and the onset risk of metabolic syndrome[80]. There are multiple experiments regarding LRP1, *in vitro* research of cell culture, and *in vivo* research of conditional knockout mouse lacking tissue-specific LRP1. Findings from these studies suggest a strong possibility that the malfunction of LRP1 is related to a mechanism of alternations from simple fatty liver to steatohepatitis.

LRP1 undergoes non-physiological epigenetic alterations. Occurrence frequency of epigenetic alternation is different, depending on location and the surroundings of gene promoters, the area of initial transcription of DNA. The promoter of the LRP1 gene, in particular, is expected to easily provide epigenetic alternations. It is reported by Dr. Nikola Barić that LRP1 is more sensitive to epigenome factors than RAGE[81]. The increase of DNA methylation inhibits the expression of the gene (localization: 12q13.3) with the decrease of the transcription, which leads to the damage in most of its functions, including the function which is related to the discharge of harmful and toxic macromolecules from the brain. The determinative factor for weakening the multiple processes is that the DNA methylation continues progression through its life cycle.

Here, we must be reminded of "what is called the phenomenon of DNA methylation, in varied research reports, includes diverse modifications such as carbonylation and alkylation". LRP1 gene promoter abundantly contains CpG island and is highly sensitive to DNA methylation. This means that LRP1 is a region where epigenetic events, such as glycative stress or excessive aldehydes, are highly expected to induce non-physiological carbonylation of DNA. With the relation to the elevation of levels in DNA methylation as well as DNA carbonylation, the transcriptive activity of LRP1 decreases. When CpG sites are completely methylated, transcriptive activity thoroughly disappears. Thus, the expression of LRP1 ceases[82].

A high-lipid diet is a primary risk factor of not solely steatohepatitis but cirrhosis and hepatoma[83]. The expression of LRP1 in hepatocyte is significant for the defense system against high-lipid-diet-related fatty liver and steatohepatitis[84-86]. On the other hand, the reduction of LRP1 expression raises the risk of steatohepatitis. Moreover, the reduction of LRP1 level is related to unfavorable prognosis of hepatocellular carcinoma[87]. The LRP1 inactivation in hepatocyte aggravates HF-diet-induced obesity, impaired glucose tolerance, insulin tolerance and fatty liver[84]. The inactivation of hepatic LRP1, with synergistic effect of dietary cholesterol, accelerates development from liver diseases to steatohepatitis[85,86]. Intensive reduction of LRP1 expression induces the hepatocellular degeneration[88].

The mechanism of the LRP1 inactivation is supposed as follows: Fat-acid-derived aldehydes, which is induced by a high-fat diet, evoke the carbonylation of cytosine in the LRP1 gene promoter domain. This leads to the decrease of LRP1 expression. This is the reason for the LRP1 inactivation.

Epigenetic alteration occurs for histone as well[11]. Acetylation/deacetylation of histone protein, in particular, lysine residue and arginine residue, are physiological modification sites. Histone acetyltransferase (HAT) activates the transcription of acetylation. Histone deacetylase inhibits the transcription via deacetylation. Furthermore, this site of histone protein undergoes modifications of methylation, phosphorylation, ubiquitylation, citrullination and crotonylation. Aldehydes induce phosphorylation and glycative modification (carbonylation) in a non-physiological manner.

Histone deacetylase (HDAC) and sterol regulatory element-binding protein (SREBP) play significant roles in maintaining the homeostasis of hepatic lipid metabolism. HDAC3 activates estrogen-related receptor alpha, induces the transcription of fatty acid transporter gene Cd36, and consequently leads to the lipid absorption and obesity[89]. Furthermore, HDAC3 knockout mouse shows the acceleration of lipid generation, the accumulation of binding substance fat-acid-derived aldehydes, and the reduction of ATP formation. Lethal phenotype is observed due to hepatic disorders[90]. Thus, the acetylation and deacetylation of histone must be strictly controlled. When histone protein is modified by excessive aldehydes, HDAC is disabled. Lipid metabolism abnormalities and hepatocellular dysfunction are expected to occur.

Rats fed a high-fat and high-fructose diet have steatohepatitis with the increased expression of HDAC3[91]. The reason for this phenomenon is the excessive aldehydes which are fatty acid- and carbohydrate-derived. The glycative modification of histone increases and the transcriptive activity is accelerated. It is supposed that the expression of HDAC3 increases as a responsive-reaction.

5. *Involvement of oxidative stress*

One of significant mechanism for the development NASH is that AGEs induce ROS on a cellular level[92,93], activating downstream NADPH oxidase via RAGE. AGEs activate hepatic stellate cells and increase the ROS formation[68]. Additionally, Kupffer cells increase the ROS formation via RAGE with the addition of AGEs[69]. These findings indicate that glycation stress (aldehyde excess) results in increased ROS production secondary to increased oxidative stress from baseline conditions. Oxidation of fatty acids by ROS induces fatty acid-derived aldehyde production, which further enhances glycation stress.

Athletes, who have been recognized to have no relationship with fatty liver, develop steatohepatitis in some cases. Exercise load is extremely heavy and oxidative damage is conspicuous in these cases. Results of a clinical test is shown regarding the healthy individuals, and the athletes, who are university student club members of *Ekiden,* Long Distance Relay, having heavy exercise load. (***Table 2***: unpublished data) The index of 8-hydroxy-deoxyguanosine is employed for the oxidative damage of DNA (constituent base guanine) and isoprostane for the oxidative damage of fatty acid. Index of 8-OHdG in the group of athletes is 10～

Table 2. Urinary oxidative stress indicators: Comparison between healthy subjects and hard-training athletes.

Subjcets		Healthy subjects	Athletes with hard training
Number		23	19
Sex		M:11, F:12	M:19
Age (year)	(year)	44.7 ± 7.9	19.8 ± 71.0
BMI		23.0 ± 2.1	21.8 ± 7.0
8-OHdG generation rate	(ng/kg/hr)	11.3 ± 6.5	119.0 ± 54.0
15-Isoprostane generation rate	(ng/kg/hr)	4.3 ± 2.8	85.6 ± 56.2
8-OHdG/Cre	(ng/mg Cre)	11.0 ± 3.8	206.6 ± 57.8
15-Isoprostane/Cre	(ng/mg Cre)	4.1 ± 1.9	1675.9 ± 1246.9

Results are expressed as mean ± standard deviation. Note that oxidative stress markers are extremely high in the athletes (members of university Ekiden Club) in season. Ekiden, long distance relay. Unpublished data.

20 times as high as that of healthy individual and isoprostane in athletes is 20～40 times as high as healthy individuals. The tests on athlete group were measured on the first day of a training camp. The values of the date are almost same as those of the day after four weeks. It is clarified that the athlete group has intensively high level of oxidative stress damage and their active oxygen and free radicals are in the excessive state. It can be said that oxidative stress contributes greatly to the development of fatty liver disease in athletes.

In hepatocyte of the athletes, lipid droplets for the storage of energy are observed. They are significant for energy source after intracellular glycogen is deleted. However, when intensive exercise load induces the excessive formation of ROS, fatty-acid-derived aldehydes form at an accelerated pace from hepatic lipid droplets. The increased MGO raises the load on GLO, and a large quantity of GSH is consumed. Thus, the function of antioxidative defense is lowered. A large quantity of AGEs, which form due to protein modification, stimulates RAGE in macrophage and Kupffer cells. Inflammatory cytokine is released, which induces inflammation. Simultaneously, the formation of collagen increases via fibroblast cells, which are stimulated glycated collagen, and Ito cells, which alters myofibroblast-like cells due to the stimulation of hepatic damage. Therefore, fibrosis progresses. It is considered that the combination of inflammation and fibrosis leads to the alternation from fatty liver to steatohepatitis.

Athletes have unique circumstances. It is known that athletes exercise at high intensity, which increases glucose use by muscles and enhances gluconeogenesis in the liver. For example, alanine is converted to pyruvate by alanine aminotransferase, which then produces glucose 6-phosphate (G6P). However, at the Anti-Aging Medicine General Meeting in May 2024, it was reported that "rapid gluconeogenesis in mice increases MGO, a short-chain aldehyde, in the liver" [94]. Although verification in humans is necessary in the future, the production of short-chain aldehydes through gluconeogenesis will require attention in the future.

Another reason is the post-exercise beverage. In addition to minerals, so-called sports drinks often contain isomerized sugar (70% or more fructose) as an energy source, rather than glucose alone. Drinking large amounts of these beverages can lead to excessive intake of fructose. In order for fructose to enter the glycolysis pathway, it must pass through fructose-1-phosphate (F1P) produced by fructokinase or fructose-6-phosphate (F6P) produced by hexokinase, and GA, a short-chain aldehyde, is produced from F1P. In fact, the aforementioned publication reported that a considerable amount of glyceraldehyde is produced in the small intestine when mice are administered fructose [94]. It has been reported that hepatitis occurs when short-chain aldehydes increase in the liver and cysteine, which reacts with aldehydes and is involved in detoxifying aldehydes, is depleted [6].

The combination of the above habits intensifies the aldehyde load and further increases the risk of fatty hepatitis in athletes.

Conclusion

Prevention and treatment for steatohepatitis

There are varied arguments regarding treatment methods. However, treatment methods for the inhibition of steatohepatitis have not been established. It is necessary to prevent progression, not neglecting it, when you are told that you have a fatty liver. Basic methods are alimentary therapy and exercise therapy. If you have obesity, diabetes, dyslipidemia and/or hyperpiesia in your background, they must be cured in every way possible.

Glycative stress (excess aldehydes) contributes greatly to the onset and progression of steatohepatitis. It is unclear at what point fatty liver and steatohepatitis become distinct. We speculate that the determining factor is whether or not there is a lack/depletion of NAD+ due to excess aldehydes, and whether or not there is a TCA cycle disorder. In particular, a malfunction of the TCA cycle causes fumaric acid modification of GAPDH, which leads to a vicious cycle of increasing aldehydes. Another key may be epigenetic glycation. In this current situation, we focused on the LTP1 gene. This issue is a future challenge.

It is important to prevent excess aldehydes through glycation care. The first step is to control aldehyde generation derived from carbohydrates, fatty acids, and luxury items, *i.e.*, alcohol, or smoking. The second step is to inhibit the glycative modification of substances in the body (*i.e.*, proteins, lipids, DNA), and the third step is to promote

the decomposition and excretion of glycation products (*i.e.*, AGEs). It is essential to fully understand the impact of glycative stress on the development of steatohepatitis and to practice glycation care.

Acknowledgments

The essential part of this paper was published in the 28th Society for Glycative Stress Research held in Tokyo in 2024.

Research Grant

This work was supported by a scientific research grant from the Ministry of Education, Culture, Sports, Science and Technology (JSPS Kakenhi 23K10882).

Conflict of interest declaration

Not applicable.

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