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REVIEW

Unknown biological effects of L-glucose, ALA, and PUFA

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Abstract Key substrates including glucose, amino acids, and fatty acids play core roles in nutrient metabolism. In this review, we describe phenomena observed when key substrates are applied to cells. We focused on three promising substrates: L-glucose derivatives, 5-aminolevulinic acid, and polyunsaturated fatty acid. Since they are assumed to give a specific reaction when they are transported into cells or metabolized in cells, they are expected to be applied in a clinical setting. We provide the latest knowledge regarding their behaviors and effects on cells.

Keywords Spheroid · Bile duct · 5-Aminolevulinic acid · Heme · Polyunsaturated fatty acid · Cell culture

Abbreviations

2-NBDG	2-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose
2-NBDLG	2-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-L-glucose
2-TRLG	2-Amino-2-deoxy-L-glucose derivative bearing either *para* or *ortho* isomer of sulforhodamine 101 acid
DAPI	4′,6-Diamidino-2-phenylindole
fLG	Fluorescent l-glucose analogue
ALA	5-Aminolevulinic acid
PPIX	Protoporphyrin IX
HO-1	Heme oxygenase-1
COX	Cytochrome c oxygenase
ROS	Reactive oxygen species
PEPCK	Phosphoenolpyruvate carboxykinase
G6Pase	Glucose 6-phosphatase
PUFA	Polyunsaturated fatty acid
CTE	Cardiac tissue equivalent
PPAR	Peroxisome proliferator-activated receptor
SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
αLA	A-linolenic acid
LA	Linoleic acid
EPA	Eicosapentaenoic acid
AA	Arachidonic acid
DHA	Docosahexaenoic acid
PKA	Protein kinase A

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Introduction

It is well known that key substrates including glucose, amino acids, and fatty acids play inherent physiological roles in nutrient metabolism. There has also been growing interest in their other physiological effects, one of which is intercellular signaling.

Therefore, in this review, we focus on cellular reactions and behaviors that are not well known when key substrates (glucose, amino acids, fatty acids, etc.) related to nutrient metabolism are applied to cells (Fig. 1). Three substrates focused on in this review, L-glucose derivatives, 5-aminolevulinic acid, and polyunsaturated fatty acid, show a unique mode of transport into cells and exertion of physiological actions unknown so far when metabolized intracellularly, which are expected to be applied to a clinical setting (Fig. 1). The latest knowledge regarding the three promising substrates is discussed.

The main text consists of three chapters and an outline of each chapter is given below.

In the first chapter, L-glucose derivatives are addressed. L-glucose is a mirror image isomer of D-glucose, which is an essential nutrient. L-glucose is rarely found in nature and neither its transportation nor metabolism has been observed in mammalian cells. However, one of the authors (K.Y.) recently discovered that some kinds of cancer cell lines show a specific uptake of fluorescence-labeled L-glucose. The author discusses a hypothetical change in the glucose transport system in cancer cells by using an L-glucose derivative as a tracer.

In the next chapter, amino acids are described. 5-Aminolevulinic acid (ALA), which is also synthesized in vivo, is a precursor of heme. It has recently been clarified that exogenous administration of 5-ALA induces various hitherto unknown biological effects. In this article, regarding ALA as a new bioactive substance, two authors (H.A., Y.M.) describe the effects that have so far been reported and discuss the possibility of application of ALA to treatments of some metabolic diseases.

In the last chapter, the unknown bioactive effects of fatty acids are described. Although it is recognized that polyunsaturated fatty acid (PUFA) plays a significant role in the adjustment of physiological functions of cells, details are unknown. In this chapter, therefore, two authors (D.S., T.N.) describe the bioactive effects of PUFA on cardiomyocytes and adipocytes and summarize the potential benefit of PUFA with respect to functional control of various cells including stem cells.

I. Visualizing cellular uptake of glucose using fluorescently labeled glucose derivatives

Background

Glucose is a fundamental fuel for most living things. Of the two stereoisomers of glucose, D-glucose, occurs widely in nature, but its mirror image, isomer L-glucose, does not. In mammals, cells are thought to utilize only D-glucose by taking it up via glucose transporters such as GLUTs [1]. To investigate D-glucose transport, radiolabeled D-glucose and its non-metabolizable derivatives such as [^{14}C] 2-deoxy-D-glucose, [^{18}F] fluoro-2-deoxy-D-glucose, and [^{14}C] 3-*O*-methyl-D-glucose have long been used [2]. These radiolabeled tracers are effective for quantifying the average uptake of D-glucose into a population of cells. However, actual tissues contain cells with divergent uptake properties both spatially and temporally, and a method for monitoring uptake at the single cell level is needed.

We have shown that 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent

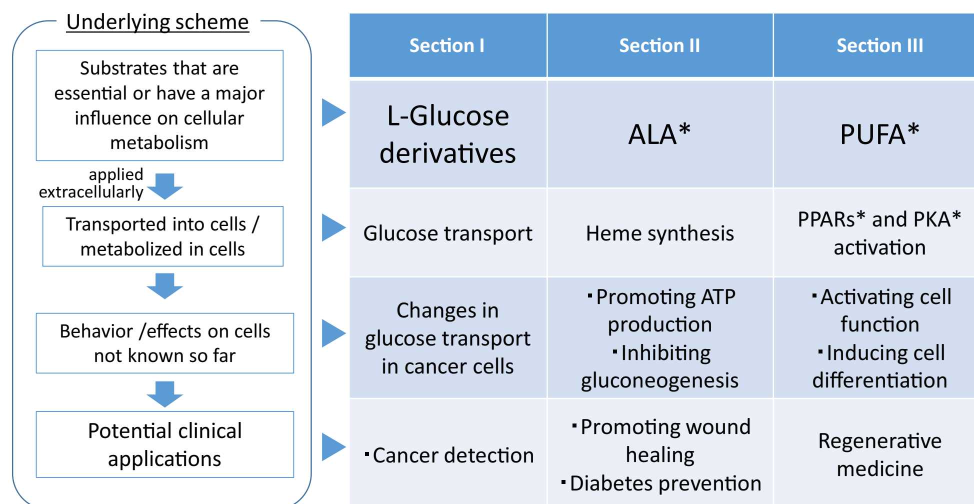

Fig. 1 Underlying scheme of this review

analogue of D-glucose [3], is taken up into single living mammalian cells through GLUTs showing K_m values similar to those reported for D-glucose and the non-metabolizable glucose analogue 3-*O*-methyl-D-glucose [4, 5]. So far, 2-NBDG has been used as a standard fluorescent D-glucose derivative for monitoring D-glucose uptake into single cells [6–9]. However, care should be taken in that fluorescence intensity is an arbitrary measure and that the uptake of 2-NBDG may vary for a short period. Quantification therefore requires an accurate procedure [5]. Indeed, precise evaluation of 2-NBDG uptake is a challenging issue, particularly when applied to thick living tissues consisting of cells showing heterogeneous activities and degenerating states. To overcome the difficulties, we developed 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaol-4-yl)amino]-2-deoxy-L-glucose (2-NBDLG), the first fluorescent L-glucose analogue (fLG), as a control substrate for 2-NBDG [10].

Although 2-NBDLG was not taken up into living *Escherichia coli* cells (data not shown), specific uptake of 2-NBDLG occurred when applied to three-dimensionally accumulating tumor cells showing nuclear heterogeneity, a cytological feature indicative of a high grade of malignancy in cancer diagnosis (Fig. 2) [11]. The effectiveness of 2-NBDLG for visualizing cancerous tissue has also been shown in a hamster model of bile duct cancer in vivo [12]. In the following sections, we discuss unique features of fLGs for investigating changes in glucose uptake properties of tumor cells.

2-NBDLG uptake occurs specifically into three-dimensionally accumulating tumor cells showing nuclear heterogeneity

A brief administration of 2-NBDG (D-form isomer) into mouse insulinoma MIN6 cells [13] resulted in an increase in cellular fluorescence when administered in early culture stages up to several days in vitro, whereas no detectable, or a negligible, increase in fluorescence was detected when 2-NBDLG (L-form isomer) was administered at the same culture stage [11]. At 10–15 days in vitro, however, remarkable uptake of 2-NBDLG occurred in tightly packed, three-dimensional spheroids (Fig. 2a) [11]. Interestingly, in an example depicted, only upper spheroids took up 2-NBDLG. To obtain more information, we applied 4′,6-diamidino-2-phenylindole (DAPI), a nuclear marker, to living spheroids. Optical sectioning at different depths demonstrated that the spheroids that took up 2-NBDLG consisted of cells with remarkable nuclear heterogeneity, an important cytological feature of cancerous cells with a high grade of malignancy (Fig. 2b, c) [11].

Three-dimensional tumor spheroids can provide valuable information on complex physiological and pathophysiological processes in vitro [14]. Quantitative analyses further demonstrated that 2-NBDLG uptake persisted in the presence of cytochalasin B, a potent GLUT inhibitor [11]. This is in contrast to the fact that cytochalasin B caused marked inhibition of the uptake of 2-NBDG (D-glucose derivative) in the same culture plate. There was no

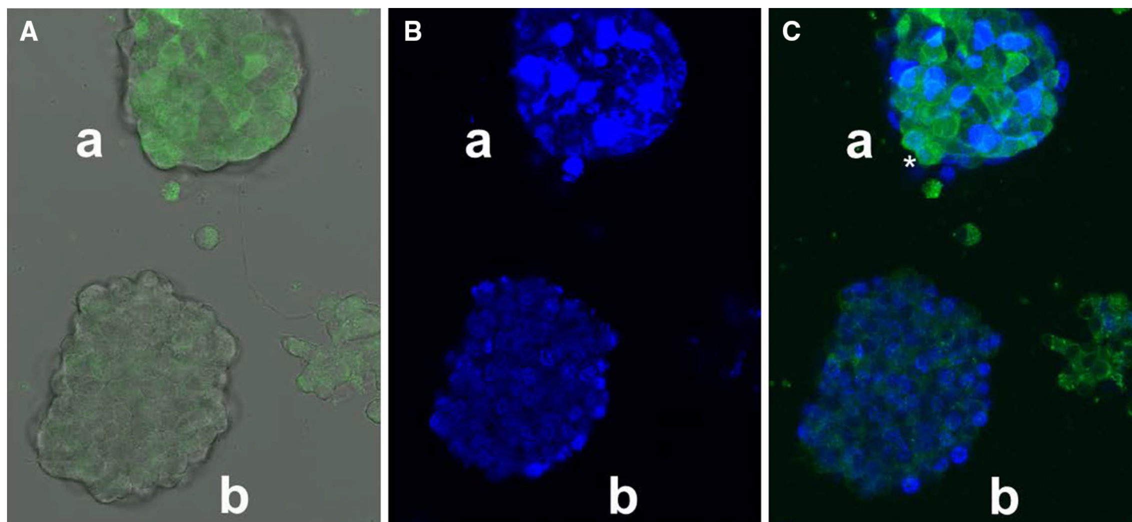

Fig. 2 Confocal microscopic images showing specific uptake of the fluorescent L-glucose derivative 2-NBDLG into mouse insulinoma MIN6 cells, which formed three-dimensional spheroids consisting of cells with nuclear heterogeneity. **a** Differential interference contrast image of MIN6 cells forming thick spheroids merged with 2-NBDLG fluorescence. Cells in the upper spheroid (*a*) exhibited strong 2-NBDLG fluorescence. **b** A single optical section of a nuclear image visualized by 4′,6-diamidino-2-phenylindole (DAPI) in a livecell condition. Cells in the upper spheroid (*a*) exhibited strong nuclear heterogeneity in morphology, whereas cells with evenly arranged small nuclei were seen in the lower one (*b*). **c** Merged image of (**b**) and 2-NBDLG fluorescence (**a**). Note that small cells bearing small nuclei (*asterisk*) also took up 2-NBDLG in the upper spheroid. Images were reproduced with permission from Sasaki A et al. [11]

significant change in the uptake in an Na^+-free condition, suggesting that SGLTs, sodium-dependent glucose transporters, are not involved in the uptake. Interestingly, phloretin, an aglycon of apple polyphenol phlorizin, abolished the uptake of 2-NBDLG [1, 15]. Phloretin is a broad-spectrum inhibitor against membrane transport including that mediated by GLUTs and water channels [11].

To further characterize the specificity of 2-NBDLG uptake, we used 2-TRLG, a Texas Red-bearing, membrane-impermeable L-glucose analogue emitting red fluorescence [16]. 2-TRLG is useful for identifying non-specific uptake of 2-NBDLG into dying cells due to, for example, loss of membrane integrity in a condition of high sensitivity [16]. The combined use of 2-NBDLG and 2-TRLG showed that specific uptake of 2-NBDLG, an L-glucose derivative, started to occur in cultured tumor cells at a certain stage around 10 days in vitro [11].

Taken together with other data, those findings described above suggest that uptake of the L-glucose derivative (2-NBDLG) occurs specifically in tumor cells in a certain advanced stage by a phloretin-inhibitable, non-transporter-mediated, yet unidentified mechanism. Some studies have shown insulin secretion from multicellular MIN6 spheroids [17, 18]. Hence, such a metabolic property of spheroid-forming insulinoma cells may also be of interest with regard to discrimination of malignant insulinoma.

In vivo imaging of bile duct cancer by fLGs

We next explored if the uptake of 2-NBDLG can be used in in vivo imaging using animal models. Bile duct cancer, or cholangiocarcinoma, is often a fatal cancer. Complete resection is currently the only potential curative treatment, especially for extrahepatic cholangiocarcinoma [19]. However, preoperative diagnoses provide only poor information on the surgical margin because the anomaly often spreads superficially along the longitudinal axis of the bile duct, making a diagnostic decision difficult. Positron emission tomography (PET) with a radiolabeled D-glucose derivative (FDG) provides only low spatial resolution images (>5 mm). In addition, discrimination of cancer and inflammation is often difficult.

Using a hamster model of bile duct cancer, we found that fLGs visualized extrahepatic cholangiocarcinoma in vivo when applied topically into the bile duct. Characteristic fLG fluorescence consisting of bright spots and dark clumps was correlated well with the area later diagnosed as carcinoma in situ or invasive adenocarcinoma [12]. Control animals showed no such fluorescence pattern.

Bacterial reverse mutation tests and extended single-oral-dose toxicity studies have been successfully completed for 2-NBDLG and 2-TRLG according to Good Laboratory Practice regulations. As such, the fLGs are promising candidates as in vivo contrast agents for detecting cancer by visualization of changes in the uptake activity of glucose derivatives, while minimizing toxicity. Clinical studies are currently underway in our laboratory to evaluate the effectiveness of fLGs for cancer diagnosis.

Concluding remarks

Fluorescent derivatives of L-glucose are unique tools for understanding changes in glucose transport of cells at the single cell level. Further study is needed for elucidating molecular mechanisms of the uptake and intracellular fate of L-glucose derivatives in tumor cells.

II. 5-Aminolevulinic acid: foe or friend to cellular metabolism?

Background

Aminolevulinic acid (5-aminolevulinic acid, ALA) is a precursor of heme synthetic metabolism. ALA has been widely used for visualization of malignant tissue by photodynamic diagnosis since it was reported in the 1980s that protoporphyrin IX (PPIX), which is an intermediate metabolite and a fluorescent substance, was accumulated in cancer cells by administering ALA [20].

The effect of exogenous administration of ALA on normal organs and tissues is not clear. However, recent studies have shown that ALA administration affects not only heme metabolic pathways but also other pathways.

Therefore, in this review, we describe the effect of exogenous administration of ALA on the heme metabolic pathway and also the effects of ALA administration on other biological metabolisms (energy metabolism, glucose metabolism, etc.), and we finally discuss what kind of biological reaction occurs in vivo.

Events induced by exogenously administered ALA

Exogenous administration of ALA, either oral administration or intravenous injection, results in the production of PPIX through heme metabolism, and, as stated above, the content of PPIX in cancer cells increases [21]. It has been reported that the accumulation of PPIX in cells depends on the intracellular iron concentration, cell cycle [22, 23], amount of mitochondria [24], and degree of differentiation of the cells [25]. It appears, however, that there is little accumulation of PPIX in normal cells compared to its accumulation in cancer cells [26]. The reason appears to be that ferrochelatase, an enzyme that catalyzes the terminal step of heme synthesis, namely the insertion of ferrous iron into PPIX, is highly activated in normal cells. As a result,

in normal cells, heme synthesis is probably enhanced by administering ALA [27]. As the heme synthesis advances, the content of free heme increases. It is therefore expected that the production of heme oxygenase-1 (HO-1), an enzyme that catalyzes the degradation of extra heme, is enhanced because the mechanism by which heme production is adjusted is in operation [28]. In fact, many studies have suggested that HO-1 is activated by ALA administration [29]. On the other hand, heme degradation by enzymes other than HO-1 has been shown in an in vitro study, but heme degradation by non-HO-1 pathways in vivo has not yet been confirmed [30]. In addition, while ALAS1 (ALA synthase) controls the amount of heme synthesis, the content of heme has the possibility of increasing [31] because the function of ALAS1 is bypassed by ALA administration.

For the above reasons, exogenous ALA administration to normal cells probably induces an increase in heme content, resulting in increased HO-1 activity.

Biological effects of ALA administration

Influence on energy metabolism

Exogenous ALA administration possibly enhances aerobic energy metabolism.

Ogura et al. [32] reported that ALA activates cytochrome c oxygenase (COX) in mitochondria, resulting in an increase in ATP. They speculated that there is a strong interaction between COX activation and heme content in cells. In addition, exogenous administration of ALA is likely to enhance energy metabolism via the upregulated TCA cycle by the usage of surplus succinyl-CoA [33], which would be used as a precursor for the synthesis of ALA if exogenous ALA is not administered.

On the other hand, there is the possibility that ALA administration damages cells. It was reported that ALA makes mitochondria swell [34], but the swelling is controlled by catalase and thiols, and thus reactive oxygen species (ROS) are more likely to be relevant [35]. In addition, ALA seems to induce DNA injuries [36]. It has also been shown that enzyme activities within mitochondria were deactivated in the muscle or liver of a rat administered ALA and that the superoxide dismutase (SOD) system was also degraded, possibly being related to mitochondrial membrane potential [37]. It was also reported that ALA administration facilitates fatigability [38].

Influence on glucose metabolism

Hara et al. reported that administration of ALA for 6 weeks reduces plasma glucose levels in rats without affecting plasma insulin levels and induces HO-1 expression in white adipose tissue and the liver. Thus, they speculated that the induced expression of HO-1 might be related to the glucose-lowering effect of ALA [39]. As described above, an increase in HO-1 suggests an increase in heme. Heme in the liver promotes complex formation of nuclear receptor subfamily 1 (Rev-Erbα) with its cosuppressor nuclear receptor co-repressor 1 (NCOR). The complex inhibits the transcription of gluconeogenic enzymes, PEPCK and G6Pase, resulting in a decrease in hepatic glucose production [31]. In other words, autonomous adjustment of glucose production in the liver may be forcibly canceled by ALA administration. On the other hand, glucose metabolism may be enhanced by the effect of ALA on adipocytes through a decrease in the amount of adipose tissue or a decrease in mitochondria within adipocytes [40].

On the basis of these findings, recent investigations have demonstrated that ALA administration would be a novel approach to prevent and treat diabetes mellitus [41]. The heme pathway of diabetic hepatocytes is more susceptible to porphyrinogenic factors [42]. In addition, the content of δ-ALA dehydrogenase is decreased in diabetic patients [43]. These findings suggest that ALA contributes to the control of blood glucose level.

Difference in the effects of ALA depending on the route of administration

Routes of ALA administration should be considered. In studies showing enhancement of energy metabolism or glucose metabolism, ALA was orally administered in most cases. On the other hand, in studies that showed degradation of metabolic activation or expression of ROS, intravenous injection or intraperitoneal administration of ALA was used. The blood concentration of ALA that has been orally administered is estimated to be less than one tenth of that in the case of intravenous or intraperitoneal administration, and thus the difference depending on the administration route may have an influence on the effects of ALA.

Future prospects

There are various reports other than those described above on the biological effects of exogenous administration of ALA. Activation of heme synthesis has been suggested to be involved in the acceleration of hair growth in mouse skin [44]. Since it has been reported that an increase of HO-1 promotes wound healing [45], the heme synthesis metabolic system probably has an influence on cellular metabolism.

Furthermore, some recent studies have shown enhancement of the effects of hyperthermia or radiotherapy

against cancers [46, 47] and the effectiveness for treatment of malaria [48].

As mentioned above, ALA induces an increase in ATP (beneficial effect) or induces mitochondrial dysfunction (adverse effect), suggesting that the amount of ALA-induced ROS production in mitochondria [35] determines the biological effect. As for ROS homeostatic pathways, mammal cells use global differentiation programs that provide either long-lasting oxidant-protective responses or cell death as clearance mechanisms for oxidatively damaged cells [49].

Heme is a core substance of hemoglobin, myoglobin, cytochrome, and catalase, and administration of ALA, a precursor of heme, is also expected to have a great influence on the synthesis of these substances. Thus, it is expected that new effects on associated biological functions will be reported in the future.

III. Beneficial effects of polyunsaturated fatty acids on cell culture

Background

In vitro cell culture using immortalized cell lines or primary cells or subcultured cells is one of the most useful methods to clarify various cellular functions. However, the results obtained from those cell lines are sometimes different from the results obtained from primary cultured cells. For instance, polyunsaturated fatty acid (PUFA), which is known as an essential fatty acid and is not synthesized in mammalians, induces cellular stress to dose-dependently decrease the viability of immortalized cardiac cells, H9c2 cells, whereas that is not the case for primary cultured cardiomyocytes derived from newborn rats [50]. Hence, primary culture might be one of the important methods for understanding cellular functions.

Primary cultured cells have sometimes been used even in clinical therapies. Injection of myoblasts or bone marrow cells has been tried for the treatment of ischemic heart failure; however, the efficacy of the therapy is limited [51]. Although cardiac tissue equivalent (CTE), e.g., reconstructed cardiac tissues with collagen gel, is expected to salvage severely damaged myocardium, its twitch stress ($\approx$2 kPa) was markedly smaller than that of the myocardium in vivo (>20 kPa) [52]. That is, CTE cannot generate sufficient contractile force to pump blood.

We previously reported that one of the causes of the difference between in vivo and in vitro cells may be insufficient intercellular connection, i.e., expression of a serum response factor (c-fos serum response element-binding transcription factor) that is involved in the processes of differentiation and hypertrophy and expression of MLC-2v, N-cadherin and connexin43, constituting the ventricle and intercalated disk, were lower in cultured cardiomyocytes than in the neonatal myocardium [53, 54].

Furthermore, we have focused on the difference in energy metabolism between mature and immature cardiomyocytes. Whereas cardiomyocytes in vivo utilize fatty acid as a main energy source [55], a conventional culture medium only contains aqueous materials such as glucose, amino acids, and minerals. Therefore, cultured cardiomyocytes are forced to use aqueous materials rather than lipids for an energy source. The fact that glucose supply and consumption in the fetal or newborn (immature) myocardium are greater than those in juveniles or adults [56, 57] suggests that cultured cardiomyocytes are too immature to acquire sufficient contraction ability.

Effects of polyunsaturated fatty acids on cultured cells

As mentioned above, hydrophobic components such as fatty acids have not generally been considered in a culture medium. In addition to the role of fatty acids as an energy source, polyunsaturated fatty acids (PUFAs) have bioactivities and are considered to be crucial ligands of peroxisome proliferator-activated receptors (e.g., PPARα and β/δ) in the regulation of lipid oxidation and storage [58, 59]. Therefore, unlike in the case of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), which cells can synthesize by themselves, PUFAs might have to be supplied from the outside.

Based on the concept described above, we compared 24 fatty acids (seven SFAs, seven MUFAs, four n-3 PUFAs, and six n-6 PUFAs shown in Table 1) in cultured cardiomyocytes, originally harvested from fetal rats, to those in the neonatal rat myocardium, and we found that contents of PUFAs in the cultured cardiomyocytes were generally higher than those in neonatal tissue, suggesting that the low twitch stress of CTE may be attributed in part to the low contents of PUFAs [60].

Considering the results, we performed primary culture of rat cardiomyocytes for 14 days in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture with supplementation of n-3 (α-linolenic [αLA, C18:3n-3]) or n-6 (linoleic [LA, C18:2n-6]) PUFA, bound to bovine serum albumin. The supplementation of 20 μM LA or 10 μM αLA increased their contents to levels close to those in the neonatal myocardium. The n-3 or n-6 PUFA supplementation also led to an increase in the contents of eicosapentaenoic acid (EPA, C20:5n-3) and arachidonic acid (AA, C20:4n-6), respectively, which are longer-chain fatty acids probably elongated from αLA or LA, although these contents were still lower than those in the neonatal tissue [61]. These results suggest that supplemented LA and αLA

Table 1 Fatty acids determined

General name	Carbon number and position of double bond
Lauric acid	C12:0
Myristic acid	C14:0
Myristoleic acid	C14:1n-5
Palamitic acid	C16:0
Palmitoleic acid	C16:1n-7
Stearic acid	C18:0
Oleic acid	C18:1n-9
Linoleic acid	C18:2n-6
γ-Linolenic acid	C18:3n-6
α-Linolenic acid	C18:3n-3
Arachidic acid	C20:0
Eicosenoic acid	C20:1n-9
Eicosadienoic acid	C20:2n-6
Dihomo-γ-linolenic acid	C20:3n-6
Arachidonic acid	C20:4n-6
5-8-11 Eicosatrienoic acid	C20:3n-9
Behenic acid	C22:0
Eicosapentaenoic acid	C20:5n-3
Erucic acid	C22:1n-9
Docosatetraenoic acid	C22:4n-6
Lignoceric acid	C24:0
Docosapentaenoic acid	C22:5n-3
Nervonic acid	C24:1n-9
Docosahexaenoic acid	C22:6n-3

were successfully incorporated into cells and that a part of them was converted into EPA or AA via elongation and/or desaturation processes. Regarding the elongation of PUFAs, Leroy et al. reported for the first time the possibility in cultured cardiomyocytes [62], while Matsuzaka et al. reported that a low mRNA expression level of fatty acyl-CoA elongase was involved in elongation of some SFAs (lauric [C12:0], mylistic [C14:0], palmitic [C16:0], and stearic [C18:0] acids) and MUFAs (palmitoleic [C16:1n-7] and oleic [C18:1n-9] acids) in mammalian heart [63].

Recently, we have tested supplementation of longer-chain AA or docosahexaenoic acid (DHA, C22:6n-3). The AA supplementation increased not only the content of AA but also that of docosatetraenoic acid (C22:4n-6) probably elongated from AA, and the DHA supplementation increased the DHA content as well as EPA content.

In addition to the changes in fatty acid contents, supplementation of PUFAs increased contractile performance severalfold in comparison to non-treated cells (unpublished data). Although the mechanisms of the elevation in contractile performance have not been clarified, it is reasonable to assume that the elevation is attributed in part to cAMP function. Intracellular protein kinase A (PKA) activated by cAMP induces phosphorylation of proteins involved in myocardial Ca^{2+} regulation [64–66], and EPA activates PKA in rat ventricular muscle [67]. Luiken et al. demonstrated that a cAMP-elevating agent increased fatty acid uptake in isolated cardiomyocytes [68]. Therefore, PUFAs may enhance their own uptake and improve contractile function via elevation of cAMP in cardiomyocytes. In clinical studies and studies using human cardiomyocytes, it has been shown that EPA- and DHA-rich fish oil supplementation induces their uptake into myocardial phospholipids and reduces cardiac mortality [69, 70].

The results described above suggest that PUFAs have important roles in the primary culture of other cells as well, probably including stem cells. For example, primary cultured adipocytes are known to become fibroblast-like cells (dedifferentiated fat cells) and then potentially differentiate into mesenchymal stem cells [71], indicating the difficulty in stable primary culture of adipocytes. Regarding the effects of PUFAs in adipocytes, some PUFAs can be ligands of PPARγ, which is dominantly expressed in adipocytes [72]. PPARγ is known as an essential factor for differentiation of adipocytes. Several studies have also suggested that not only a PPARγ agonist but also EPA and DHA induce “browning” of adipocytes (conversion of white adipocyte into brown adipocyte) in vivo and in vitro [73–75]. Therefore, PUFAs could enable artificial control of adipocyte function in culture. We have just commenced preliminary experiments based on this concept.

Conclusions

It is thought that supplementation of PUFAs may be essential in the culture of various cells except for immortalized cell lines. The supplementation potentially has various beneficial effects on biological functions such as glucose and/or lipid metabolism, differentiation, and maturity of cultured cells. Further studies are needed to optimize the amount and combination of supplementary fatty acids for enhancing or maintaining cellular function.

Conclusion looking over the three substrate topics and perspective about future advances of key substrate research

The aim of conventional research on a key substrate was elucidation of the physiological behavior in its metabolic (synthesis, degradation) pathways or an understanding of pathological states caused by the impairment or failure in its pathways. In other words, a key substrate was treated as “a constituent element” of the pathway in most studies.

However, the approach in studies introduced in this review was to consider a key substrate as an active substance and to observe the reactions elicited in cells when it is administered. Namely, the research method resembles that of pharmacology. Although such a research method is not new, it may lead to information that has not been obtained so far and it may be used in parallel with a diversity of research technology.

Compliance with ethical standards

Conflict of interest One of the authors (K.Y.) declares the following competing financial interest(s): The author received grants from the Japanese government for developing potential cancer diagnostic agents, including Science and Technology Incubation Program in Advanced Regions, Collaborative Research Based on Industrial Demand, and A-STEP from JST, and is an applicant for multiple patents including WO2012/133688 with Peptide Institute, Inc. The author assigned ownership of the patent to Hirosaki University. The other authors (D.S., T.N., H.A., Y.M.) declare that they have no competing interests.

Ethical approval In a part of this study (Section I), all animal studies were performed in accordance with and approved by the Animal Care and Use Committee of Hirosaki University Graduate School of Medicine. All procedures performed in studies involving human participants were approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine and were in accordance with the 1964 Helsinki Declaration and its later amendments. In another part of this study (Section III), all of the experimental procedures were approved by the Yamagata University Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health.

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