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THE JAPAN ENDOCRINE SOCIETY *SINCE 1925*

ESSAY | TOWARD JES 100TH ANNIVERSARY: REMARKS FROM HONORARY MEMBERS

A 40-year challenge to resolve clinical questions through experimental/laboratory techniques

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Introduction

Throughout my clinical work in endocrinology and metabolism, I frequently encountered a variety of unresolved phenomena, that sometimes yield biologically important findings. In most cases, however, questions cannot be completely answered by clinical examination alone. As a physician-scientist, I have tried to answer these questions using physiological and molecular biological techniques, with the support of many colleagues and through academic activities in the Japan Endocrine Society. In this essay, I aim to share insights from my 40-year career in research, including both published and unpublished data, which I hope might be helpful to young endocrinologists in their future research.

Neurohypophysis

When I started my work as a clinical endocrinologist in the early 1980s, various provocation tests were already available to estimate the function of the anterior pituitary gland. In contrast, the function of the posterior pituitary gland remained a black box, due to the lack of a sensitive vasopressin (AVP) assay. We had been using urine osmolality as a surrogate marker during water restriction and AVP loading tests, which imposed a burden on patients. Fortunately, a new AVP assay with high sensitivity and specificity became available in the mid-1980s. Dr. Yutaka Oiso, my mentor at Nagoya University, and I were involved in developing a new provocation test using this new assay and a 5% hypertonic saline infusion, which could clearly identify patients with central diabetes insipidus (CDI) [1]. This assay also allowed us to determine urine AVP, aiding in the diagnosis of CDI [2].

Using this assay, I examined various patients with suspected neurohypophyseal dysfunction, and identified the following:

1) Both the posterior and anterior pituitary glands are compromised in Sheehan's syndrome [3].
2) Hyponatremia in myxedema patients is AVP-independent [4].
3) Hyponatremia in patients with adrenal insufficiency is either AVP-dependent or AVP-independent [5].
4) AVP hypersecretion is found in patients with uncontrolled diabetes mellitus (DM) [6].
5) Subclinical AVP hyposecretion is found in myotonic dystrophy [7].
6) Preexisting subclinical central or nephrogenic DI can cause overt polyuria during late gestation and parturition in patients with transient DI of pregnancy [8].

In parallel with my clinical work, I started physiological experiments to elucidate the neuroendocrinological regulation of AVP secretion. Analgesic use of morphine, a μ-opioid agonist, was frequently linked to antidiuresis in patients, suggesting the involvement of opioid peptides in the regulation of AVP secretion. In contrast, Dr. Oiso reported a marked diuretic effect of opioid κ-agonist [9]. Under the mentorship of Prof. Gary L. Robertson in the neuroendocrine department at the University of Chicago, I began examining the effects of various opioid receptor ligands on AVP secretion in rats [10, 11] and eventually identified their site of action in the brain [12]. We also characterized the resetting of osmolality-AVP relationship in chronic hypovolemia, a condition frequently encountered in clinical settings [13].

Unfortunately, I developed a rat allergy, which made it difficult for me to continue animal experiments. Coincidentally, in the early 1990s, molecular biological technologies, including PCR, became available, and I embarked on a new challenge to acquire experimental skills. Under the mentorship of Prof. Joseph Majzoub at Harvard Medical School and Boston Children's Hospital, I started cloning and characterizing rat and human AVP genes and promoters. We found that the cAMP/PKA/

CREB pathways enhance AVP gene transcription, while glucocorticoid inhibits it [14, 15]. Later, Prof. David Murphy and Prof. Michael P. Greenwood at the University of Bristol expanded on this research *in vivo*, showing the role of CREB3L1, among the CREB family proteins, in AVP gene transcription [16]. In my collaboration with them, we found that CREB3L1 also mediates the expression of PC1/3 processing enzyme gene in response to osmotic stimuli, establishing the new concept that post-translational processing is another regulatory step in the osmolality-induced generation of mature AVP peptide [17]. In addition, Dr. Masanori Yoshida, a colleague in my research group in Japan, and I further characterized the AVP gene promoter, and revealed the crucial role of AP1 for positive regulation [18].

While I was at Prof. Robertson's lab, he was characterizing several large families suffering from familial central DI (FDI) of unknown etiology. Although we were very interested in their genetic background, the etiology remained unresolved. At the 1990 International Vasopressin Conference held in Montpellier, I was very surprised to hear Dr. Hartwig Schmale and Prof. Dietmar Richter, molecular biologists from Germany, give their first presentation about the mutation of the AVP gene in FDI patients (a Gly to Val missense mutation at position 17 in highly conserved neurophysin) [19]. Since many other neurophysin mutations were subsequently reported in different FDI families, I tried to examine the impact of the mutant AVP gene expressed in differentiated neuronal cells and found that multiple inclusion bodies gradually emerged in the cytoplasm, followed by cell death [20, 21]. I also tested the effect of expressing AVP genes harboring artificial mutations in the neurophysin. The results showed that AVP secretion was generally diminished in all the mutant genes, suggesting the important role of the neurophysin protein in maintaining appropriate AVP secretion [22].

One of the most intriguing clinical questions in my career has been the mechanism of hyponatremia in adrenal insufficiency. This issue has a long history from the 1960s, since the debate between Prof. Joseph F. Dingman (AVP-dependent) and Prof. Charles R. Kleeman (AVP-independent). Current understanding suggests both mechanisms are present depending on the clinical situation. Indeed, I witnessed a case of pre-existing CDI (AVP-deficient) that subsequently developed adrenal insufficiency with hyponatremia, supporting the AVP-independent mechanism [5]. What, then, is the AVP-independent mechanism? Glucocorticoid (GC) is known to regulate the activity of Na-Cl cotransporter (NCC) [23]. NCC plays an indispensable role in diluting urine within the kidney's distal tubule, and inhibition of NCC with thiazide diuretics frequently causes hyponatremia (known as thiazide-induced hyponatremia). Thus, GC deficiency in adrenal insufficiency may cause NCC hypofunction, resulting in impaired water diuresis and hyponatremia. In addition, I propose two hypotheses to further explain this mechanism.

1) The expression and activity of many ion channels and transporters are dependent on the presence of GC (as described later). Therefore, GC deficiency can cause a failure to maintain the differential sodium concentration between intra- and extracellular spaces. Theoretically, in this situation, sodium may shift between these two compartments, causing extracellular hyponatremia.
2) Intracellular osmotic equilibrium is maintained by the presence of osmogenic substances including taurine. The rate-limiting enzyme in taurine synthesis is cysteine-dioxygenase 1 (CDO1), the expression of which is GC dependent [24]. Therefore, GC deficiency could theoretically impair taurine synthesis, resulting in decreased intracellular osmotic activity within the osmosensory neurons in the hypothalamus, thereby lowering the set point of systemic osmoregulation.

I sincerely hope that future research by young researchers will either prove or disprove these two hypotheses.

In CDI therapy, no significant advancement has been recognized since the development of DDAVP. To achieve a long-term antidiuretic effect, Dr. Yoshida and I created an animal model for gene therapy using electroporation of a furin-processible mutant AVP expression vector into skeletal muscle. This strategy was successful, and achieved a significant improvement of polyuria for over 3 weeks [25]. To the best of our knowledge, this is the first successful gene therapy for CDI and a pioneering work in gene-therapy-based peptide replacement, awaiting clinical application.

CRH-ACTH Axis

Elucidation of the molecular mechanism of stress remains an urgent research theme in modern society. During my time at Prof. Majzoub's lab in Boston, one of my colleagues was trying to construct a CRH knockout mouse, which piqued my interest in the regulation of the CRH-ACTH axis. Since the pivot of the HPA axis is POMC/ACTH, I developed a ~1 kb of the rat *POMC* gene-promoter-reporter construct and stably introduced it into the murine corticotroph adenoma cell line (AtT20). Cultivated in a low-serum medium, the AtT20PL cell line demonstrated strong responsiveness to CRH and AVP, indicating that it maintained the original characteristics of authentic corticotroph cells. Using this cell line,

my colleagues and I confirmed that CRH effects on POMC induction and ACTH release were mediated by cAMP/PKA and calcium (Ca) signaling pathways [26, 27]. Interestingly, various immunological and metabolic stimuli, such as LPS/innate immunity, hyperglycemia/ROS, and starvation/AMPK activation, also induced *POMC* transcription [28-30]. In contrast, GC showed marked inhibitory effects on *POMC* transcription through both genomic and non-genomic mechanisms. In the latter case, I unexpectedly found that the inhibitory Gi/o protein was involved in the rapid suppressive effect of GC [31]. In the former case, the GC inhibition was attenuated in the presence of ROS [32]. This may partly explain the clinical phenomenon that ACTH release is maintained despite high GC levels during severe inflammation. In addition, we observed that local/paracrine peptides such as PACAP/VIP and other bioactive substances and drugs also affect *POMC* transcription [33-38].

A particularly interesting finding was the effect of inflammatory cytokines (IL-1β, TNFα) on *POMC* expression: each cytokine alone showed minimal effect, whereas they significantly potentiated the positive effect of CRH [39]. This may suggest the presence of a crosstalk mechanism whereby cytokine signals somehow amplify the GPCR- or other types of receptor-mediated effects. In clinical situations, we sometimes observe enhanced sensitivity to various chemical or physical stimuli under inflammatory conditions, which could be explained by the booster effect of cytokines.

We also characterized the transcriptional regulation of CRH, a key hormone of the HPA axis, using the BE(2)C neuronal cell line in which CRH is endogenously expressed. Regarding positive regulation, we identified a major role of the calcium/CaMKIV/CREB pathway [40]. However, the molecular mechanism of the negative feedback effect of GC remained elusive, as the previously reported negative GRE was nonfunctional under our experimental conditions, and the roles of inhibitory cofactors harboring HDAC activity were not identified [41].

Cushing's disease (ACTH-producing PitNET) typically shows hypersecretion of ACTH and cortisol. A unique characteristic of this disease is that inhibition by dexamethasone is diminished but not abolished (Fig. 1). Why does the inverted sigmoid curve in this figure shift to the right? We hypothesized that the pathway of cortisol catabolism, specifically, the conversion of active cortisol to inactive cortisone mediated by 11β-HSD2, may be enhanced. To prove this, we pharmacologically inhibited the enzyme. As expected, the insufficient suppression by corticosterone was reversed [42], suggesting that enhanced 11β-HSD2 expression might be responsible for the weak suppression in corticotroph tumor cells. Supporting this hypothesis, a subsequent study has reported that the expression level of 11β-HSD2 mRNA in functional human corticotroph tumor cells is higher than that in non-functional corticotroph tumor cells [43].

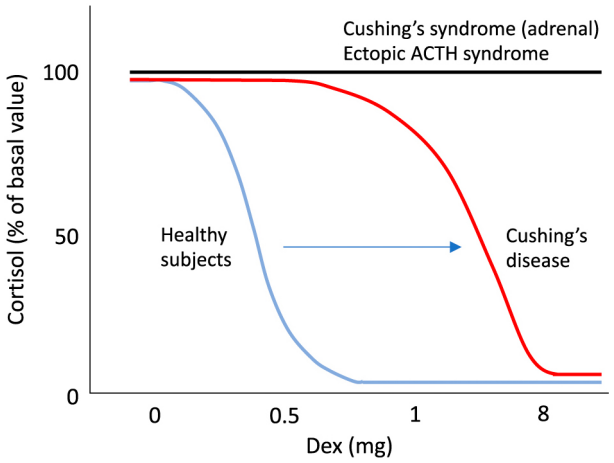

Fig. 1 Differential cortisol responses to exogenous dexamethasone administration in healthy subjects or in various types of hypercortisolism (conceptual figure, created by Iwasaki Y.)

Adrenocortical Steroids

Cortisol excess characterizes Cushing's syndrome. Why do fat accumulation and obesity occur in this syndrome? After moving to Kochi Medical School, under the guidance of Prof. Kozo Hashimoto, I tried to resolve this simple clinical question. We cloned the 5'-promoter regions (~2 kb each) of virtually all lipogenic genes and examined the effect of glucocorticoid. Our findings revealed that the gateway genes of fatty acid synthesis (ACC1, 2) and most of the genes involved in triglyceride synthesis were potently induced by dexamethasone [44, and unpublished data]. Since glucocorticoid is originally a starvation hormone, we interpret that the hormone, during predation, converts surplus carbohydrates to lipids as energy storage for survival during famine conditions. However, pathologically high plasma cortisol in Cushing's syndrome causes excessive fat accumulation regardless of nutritional condition.

We also investigated why central obesity occurs in metabolic syndrome. We found that inflammatory cytokines, known to be produced in the inflamed adipose tissues and Kupffer cells in the liver with MASLD, potently induce the 11βHSD1 gene [45], which is further enhanced by concomitant high insulin and glucocorticoid [46]. Thus, we speculate that inflammation-induced 11βHSD1 facilitates a high intracellular cortisol state, which in turn causes intracellular fat accumulation in

adipose tissues and the liver (intracellular Cushing state) [46]. We also examined the molecular mechanism of glucocorticoid resistance, focusing on GRβ isoform. We clarified that GRβ exerts a dominant negative effect, inhibiting GRα's trans-repressive effect on inflammation-related gene induction [47].

In adrenal insufficiency, hypotensive shock frequently occurs, likely involving ion channel and transporter genes. To investigate this, we analyzed the effects of various ion channel/transporter genes expressed in vascular smooth muscle cells (VSMC) using the A10 rat VSMC cell line. We found that most of the ion transporter genes examined (Na-K ATPase subunits, ENaC, NKCC, NCC, *etc.*) were glucocorticoid sensitive (unpublished). This suggests that, without GC, VSMCs lose their excitatory capacity to maintain vascular tone, leading to vascular collapse. Conversely, excess cortisol may cause hyperexcitation and hypersensitivity to vasoconstrictive stimuli, resulting in hypertension in Cushing's syndrome.

Another representative adrenocortical steroid is aldosterone (aldo), an excess of which causes primary (or secondary) aldosteronism. While glucocorticoids can activate both GR and MR, aldosterone specifically binds to MR. To precisely understand the molecular mechanism of MR-mediated transcription, we examined the effect of aldo in GR-deficient cell lines [BE(2)C or T84]. Unexpectedly, aldo showed no effect when only MR was expressed, but exerted MR-target gene induction when both GR and MR were expressed [48]. In other words, GR plays an indispensable role in aldo-stimulated MR-mediated transcription. This unexpected concept was overlooked for a long time, but recent studies have shown results supporting this model [49, 50].

In addition, the adrenal cortex also produces DHEA, a precursor of androgen and estrogen. However, it is not known whether DHEA has an independent direct effect *via* a specific receptor, and it is sometimes called a "mystery hormone." I tackled this issue by examining the effect of DHEA on the transcription mediated by various nuclear receptors, but without conclusive results. Occasionally, however, I found that DHEA exhibits an anti-inflammatory effect as it potently inhibited cytokine or H_2O_2-induced NF-κB-dependent transcription [51]. Thus, DHEA has antioxidant properties, which may, at least in part, explain its pleiotropic effects related to anti-aging properties.

GRH-GH-IGF-I axis

Although growth hormone (GH) and prolactin are both anterior pituitary hormones, they are structurally cytokines and indeed act on receptors belonging to the class 1 cytokine receptor superfamily. Furthermore, GH exhibits both somatotropic (*i.e.*, anabolic) actions with IGF-I and catabolic actions for survival during starvation. To understand the apparently antithetical action of GH, we cloned and characterized the regulatory regions of GRH, GH, and IGF-I genes. We have now recognized the essential biological roles of GH in vertebrate evolution, as described in our recent review [52].

Thyroid

My first mentor was Dr. Yoshimasa Shishiba at Toranomon Hospital, the first chairman of the Japan Thyroid Association. During my clinical training under his direction, I had a simple but unresolved question: What is the molecular nature of hypermetabolism in patients with Graves' disease? Because thyroid hormone acts through nuclear receptor TRs, I stated cloning various genes containing TRE within their promoter regions. Among the genes tested, Dr. Lifeng Zhao, a doctoral student, and I found that GPDH and GPAT3 are T_3-inducible genes [53]. GPDH, along with malic enzyme (a well-known target of T_3), is responsible for the production of NADPH, a power source of reductive reaction in carbohydrate and lipid anabolism. GPAT3 is one of the enzymes involved in triglyceride synthesis. In collaboration with Dr. Kazuhiro Kageyama at Hirosaki University, I also found that adenine nucleotide translocator (ANT) genes respond to T_3 (unpublished). Because ANTs are mitochondrial ATP transporters, their increase allows the efficient transport of mitochondria-generated ATP to the cytosol, where most ATP-dependent molecular events (*i.e.*, enzymic reactions, receptor/transporter functions, etc.) occur. We thus speculate that the nature of thyrotoxicosis is an intracellular "ATP-toxicosis" where both anabolic and catabolic reactions are excessively enhanced.

Parathyroid

While working in the pathology department at Toranomon Hospital, I learned from Dr. Hiroshi Matsushita, an endocrine pathologist, that the relationship between plasma calcium (Ca) and parathyroid hormone (PTH) secretion shows a reverse sigmoid curve. Later, I joined the endocrinology division of Nagoya University under the direction of Dr. Akio Tomita, a pioneering researcher on the parathyroid gland. I wanted to develop a new clinical test to evaluate residual parathyroid function, similar to provocation tests for anterior pituitary hormones, because the outdated EDTA-based test had serious side effects such as hypotensive shock. After much thought, I came up with an idea for a new provocation test using sodium bicarbonate (SB) (MEYLON 8.4%®), which is routinely used in clinical settings.

As expected, the SB-induced decrease in plasma ionized calcium produced a remarkable and prompt PTH response without significant adverse effects [54] (Fig. 2). This test was subsequently found to be useful for the diagnosis of various parathyroid diseases, such as subclinical hypoparathyroidism, hyperparathyroidism, Mg deficiency-related parathyroid disorder, and HDR syndrome [54-56]. Furthermore, this test was also applicable to mice, and was useful for the evaluation of parathyroid organoid function [57].

We also carried out basic research in the parathyroid field. Using the PT-r cell line derived from the rat parathyroid gland, we examined PTH gene transcription, and identified the role of GCMB in the tissue-specific regulation of the PTH gene [58]. Notably, knockout or loss of function mutation of GCMB is reported to result in hypoparathyroidism [57, 59]. In addition, another transcription factor, GATA3, is also involved in PTH gene expression. Drs. Yoshida, Tetsuji Okawa and I identified a novel GATA3 mutation in HDR syndrome associated with hypoparathyroidism [60].

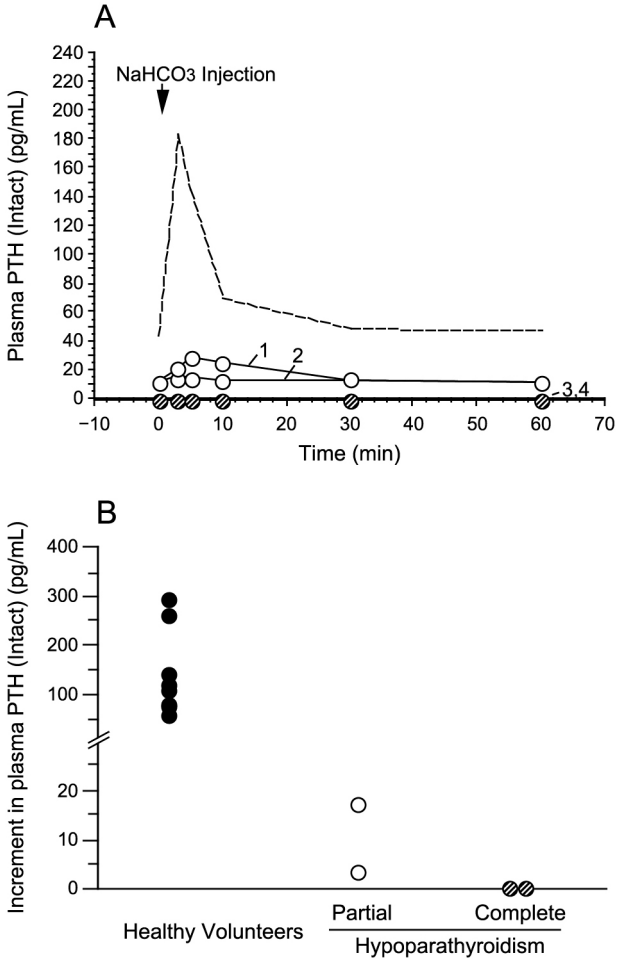

Fig. 2 Effects of sodium bicarbonate infusion on plasma PTH levels in four patients with primary hypoparathyroidism. Panel A shows the time course of the plasma intact PTH concentration in each patient (1–4) during the test. Dotted line shows the mean response in healthy volunteers. Panel B shows the absolute increment in plasma intact PTH (peak value minus value at time zero) in patients (right, middle) and in healthy volunteers (left). (Ref. 54).

Clinically, I had a simple question: why does deficiency in prohormone 25(OH)D, not $1,25(OH)_2D$, result in metabolic bone disease (hypovitaminosis D)? [61]. To explain this phenomenon, I hypothesized that 25(OH)D acts as a negative regulator of PTH *via* its conversion to bioactive $1,25(OH)_2D$ within parathyroid cells. Indeed, we found that 1α-hydroxylase (1αOHase) is expressed in PT-r cells, and at physiological concentrations, 25(OH)D rather than $1,25(OH)_2D$, suppressed PTH gene transcription [62]. Furthermore, the 25(OH)D-mediated inhibition was abolished in the presence of a 1αOHase inhibitor, strongly suggesting that 25(OH)D is a physiological regulator of PTH expression [62], the deficiency of which causes hypovitaminosis D with secondary hyperparathyroidism. This concept has recently been shown to also apply to the human parathyroid gland [63].

I was also interested in the molecular mechanism of calcium receptor (CaSR)-mediated PTH inhibition. Plasma ionized calcium binds to parathyroid CaSR and suppresses PTH expression. In this process, while CaSR is linked with Gq/G_{11} resulting in an increase in intracellular Ca [64], PTH gene transcription is somehow suppressed. Based on our preliminary results and in collaboration with Prof. Takashi Nakamura at Tohoku University, we proposed a highly plausible hypothesis in which intracellular calcium-mediated activation of NFAT induces epiprofin (specificity protein 6; Sp6), which competes with positive transcription factor Sp1 and exerts a negative effect (Fig. 3). This work is currently in press [65].

Diabetes

When I started seeing patients with diabetes mellitus (DM), the 100 g or 75 g oral glucose tolerance test (OGTT) was used for the evaluation of β-cell function. However, it was risky to perform OGTT on hyperglycemic patients. Moreover, the obtained results (*i.e.*, insulin secretory response to hyperglycemic stimuli) were apparently low and did not reflect true β-cell function due to glucose toxicity. Since the glucagon stimulation test was developed at that time, I examined whether glucose toxicity affected insulin responses to glucagon stimuli in untreated DM patients. The results showed that insulin responses to glucagon were not influenced by hyperglycemia [66]. Thus, this test proved to be a reliable tool to judge the necessity of insulin therapy, even

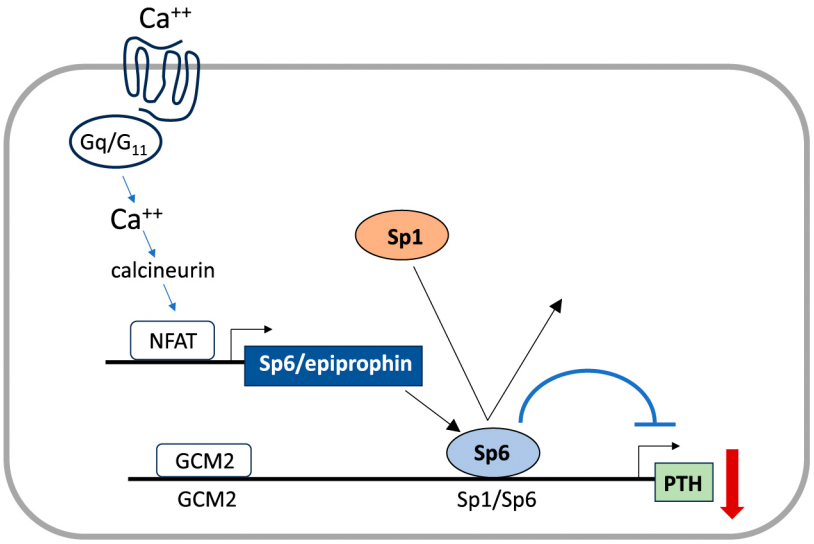

Fig. 3 Hypothetical model of hypercalcemia-induced PTH inhibition.

in naïve DM patients with hyperglycemia [67].

I was also curious about a simple question: why does excessive carbohydrate intake lead to obesity? In hepatocytes, incorporated glucose flows into the glycolytic pathway, with PFK2/FBP2 serving as a key enzyme regulating glucose metabolism. Dr. Zhao and I found that the PFK2/FBP2 gene is regulated by the nuclear receptor and transcription factor LXRα [68], which is recognized as an intracellular glucose sensor [69, 70] and regulates SREBP1c, the master transcription factor of lipogenesis. Thus, we clarified how excessive carbohydrates flow into glycolytic and then lipogenic pathways under the control of LXRα. Dr. Zhe Wang, a master's student, and I also reported that the transcription of glycolytic enzyme genes and pyruvate dehydrogenase kinase/phosphatase genes is controlled by various hormones (glucocorticoid, insulin, glucagon/cAMP) in hepatocytes [71].

Finally, I also recognized that both insulin and glucose are directly involved in inflammation-related diabetic complications [72, 73].

Aging

As our country transitions into an aging society, I have realized the importance of research focused on healthy longevity. Because SIRT1, a member of the sirtuin family, is recognized as one of the key molecules for longevity, we examined the transcriptional regulation of the human SIRT1 gene and found the involvement of nuclear receptor PPARβ/δ [74]. Since intracellular fatty acids serve as ligands for this receptor, we speculate that food restriction-mediated lipolysis facilitates PPARβ/δ-mediated SIRT1 expression, which in turn deacetylates and activates a variety of cell survival-related proteins including FOXO1 and PGC1α.

Conclusion

As mentioned in the introduction, new biological findings are sometimes obtained from naïve questions about trivial but unusual patient phenotypes. In my case, I was fortunate that I could pursue and resolve some of my questions using physiological, pharmacological, and molecular biological techniques, with significant support from my colleagues. From my standpoint, it is unfortunate that young physicians today tend to skip or hesitate to do basic research, missing opportunities to obtain new findings. I believe that findings obtained from various patients will be a major driving force behind medical progress.

Acknowledgement

I would like to thank Dr. Takako Araki for her helpful suggestions.

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