COMMEMORATIVE LECTURE

PPARs and the complex journey to obesity

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I would like to thank all of you for staying through this long day of lectures, as I get to have the last word. I wish to thank Drs. Hayashi and Kizaki for their kind remarks and for keeping everyone on time and to also thank Dr. Miyashita, my co-recipient of this award for staying until the end of these talks. We have had two interesting sessions, one that has dealt with cognition and neuroscience, and another that has dealt with the molecular biology of lipids and hormones. In some respects, each of these can be thought of as a separate science, which in fact they are. But from another perspective, all disciplines are linked and we have every reason to believe that nuclear receptors and cognition will one day intersect. Why? In part because nuclear receptors are expressed in every region of the brain and hormones affect behavior in quite profound ways from reproductive behavior to the development of the brain itself. Nuclear receptors are widely expressed in neural stem cells, differentiated neurons, and glial cells. Thyroid hormone, glucocorticoids, and retinoids are major regulators of neural activity. Thus, at each of these levels and from various perspectives, convergence between these various systems can be found.

The Nuclear Receptor Superfamily

Today I am going to talk about a segment of the nuclear receptor superfamily. At present in our genome, based on sequence and what has been isolated, there are 48 genes that encode a nuclear receptor (for review, Mangelsdorf *et al.*, 1995).[1] These 48 receptors comprise what we call a ''superfamily'', and this entity can be thought of as the combined functions of the individual receptors. We can look at each receptor individually, or take a more global view of their functions as a matrix or network in which they are not really separate, but are interconnected. While we can study individual receptors, we need to keep in mind that there probably is a matrix that connects the entire family, and thus there is a higher principle that is operating at the genetic level.

To briefly summarize, nuclear receptors have a central DNA binding domain (DBD) and a carboxy-terminal ligand binding domain (LBD) (Fig. 1). The DBD allows them to bind to and activate target genes, thus defining them as transcription factors. The LBD modulates their activities, making them hormone-dependent transcription factors. They act as genetic switches that are controlled by ligands.

Nuclear Receptor Subclasses

The receptors can be grouped into several classes as shown in Fig. 1. One class binds endocrine hormones such as the adrenal and gonadal steroids. These include glucocorticoids and mineralocorticoids that regulate sugar and salt levels in the body, as well as the sex steroids estrogen, progesterone, and androgen. Another class comprises receptors for vitamin A derivatives, thyroid hormone, and vitamin D. These compounds are involved in activities that range from neural differentiation, treatment of leukemia, regulation of basal metabolism, and regulation of calcium absorption and bone mineralization. Together these receptor systems play important roles in controlling organ physiology via transcriptional mechanisms that differ from that of cell surface receptors. Then there are the orphan receptors which comprise the remainder, and by number the largest part of the superfamily (Mangelsdorf and Evans, 1995).[2] These are members that were identified first by homology as genes and their existence led us to search for new ligands. When ligands are found for these orphan receptors, we take them out of the ''orphanage'' and adopt them. I will talk about some of these adopted orphan receptors, specifically, the PPAR subfamily, in

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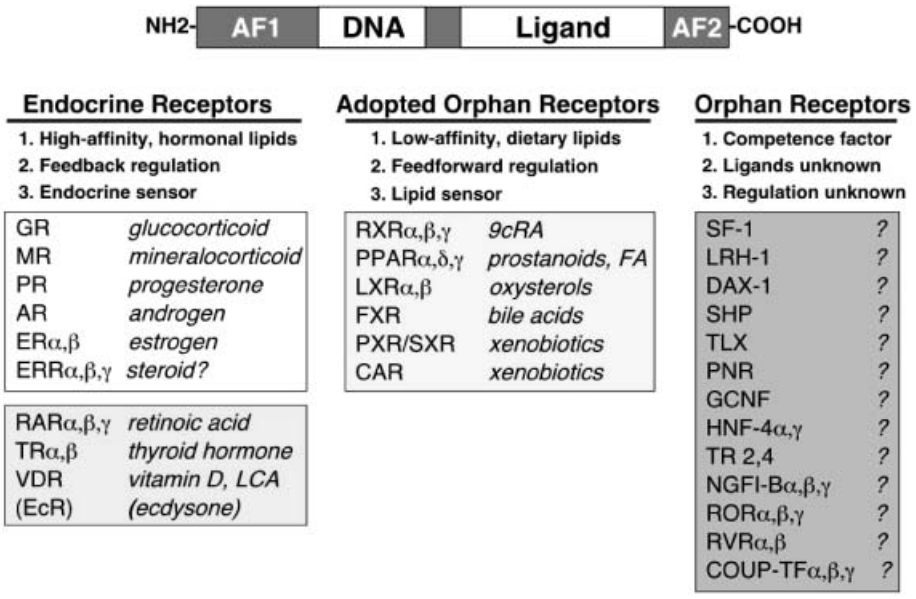

Fig. 1 Nuclear receptor superfamily.

more detail. I would like to mention two properties of these adopted orphans: one, they form heterodimers with the retinoid X receptor (RXR), and two, they respond to dietary compounds such as lipids, that is, molecules that we take orally. This has opened up entire new areas of endocrine physiology.

"Reverse Endocrinology"

Next I will describe a strategy called ''reverse endocrinology''. Forward endocrinology is where you start with the hormone and then search for the receptor. For example, we used estrogen to find the estrogen receptor. Reverse endocrinology consists of first finding the receptor and then looking for the hormones (Kliewer *et al.*, 1999).[3] For the orphans, our approach has been to search for ligands, which have turned out to be dietary lipids that include oxysterols and bile acids, both of which are cholesterol derivatives, and fatty acids and xenobiotic lipids in drugs.

Because these orphan receptors are transcription factors, we looked for their target genes and what we found were genes that are involved in lipid detoxification, transport and storage (Chawla *et al.*, 2001).[4] These include cytochrome P450s, the ABC cholesterol transporters and cytosolic binding proteins that transport lipids. We then used genetics, transgenics or knockouts to characterize their various phenotypes and again we observed problems with lipid homeostasis that affected cholesterol, bile acid, fatty acid and drug metabolism. So the orphans appear to play a central role in body physiology.

I will describe only briefly the mechanism by which the receptors work, as this has already been covered in the earlier talks. The receptors are a relatively small part of the overall complex that we believe comprises more than 75 or 80 proteins that are involved in transcriptional control (Collingwood *et al.*, 1999).[5] The retinoic acid receptor, in the absence of a hormone, is a repressor and it collects a series of repressor proteins like a magnet. Addition of the ligand reverses the polarity of the magnet, causing the repressors to fly off and the coactivators to bind, resulting in chromatin modification to activate transcription. This is how the hormonal or physiological signals affect the recruitment of co-factors that modify the chromatin in target genes. Every cell has receptors and therefore can respond, but each cell responds in its own unique way, and thus the same hormone can have a different effect in a neuron versus an epithelial skin cell versus a bone cell. In this way each receptor has a genetic network and it is the combined network that leads to the physiological effect in the body. Each of the 48 genes has its own network, so there is a very complicated set of pathways.

PPARs and Disease

Next I will focus on two of the PPAR receptor systems (Lee *et al.*, 2003).[6] They both respond to dietary lipids and are involved in lipid metabolism. They both form heterodimers with RXR and in response to their ligands, control the activity of a set of target genes. That being said, these two very closely related receptors function in opposing ways in controlling lipid me-

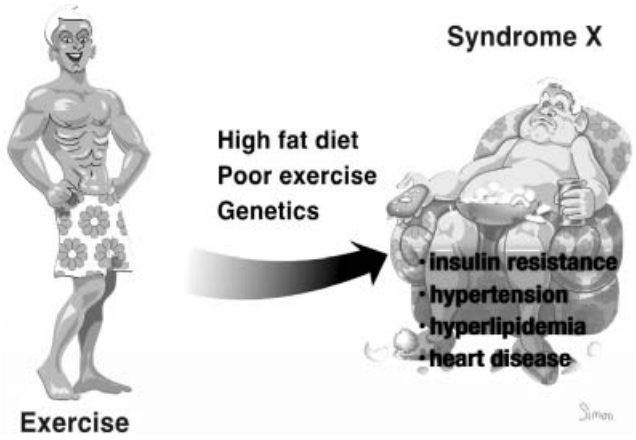

Fig. 2 PPARs and heart disease.

tabolism, which I will talk about in more detail, particularly as it relates to obesity in obesity-related diseases such as heart disease and insulin resistance.

How are the PPARs involved in heart disease? In the California surfer view of this process, we start out young, blond, slim and with exercise, we look good; then through the combination of genetics, a high-fat diet, and little or no exercise as we get older, we evolve and ''expand'' into a more mature state (Fig. 2). Instead of surfing in a bathing suit, we sit on the couch and surf channels on television. This decrease in exercise leads to an obesity-related problem called Syndrome X, that includes insulin resistance, hypertension, hyperlipidemia and heart disease. Our society is facing the problem that aiming the remote control for the television has often become the most exercise many ''couch potatoes'' get.

The reality is that Syndrome X is an obesity-related disease (Haffner and Taegtmeyer, 2003).[7] Sixty-five percent of Americans are overweight, which is the leading risk factor for Syndrome X. The United States is approximately three billion pounds overweight, which is the basis for the obesity epidemic. We are continually getting heavier and this excess weight carries a major health problem to society. The best treatment for obesity is diet and exercise, but even though that advice is repeatedly given, it is generally not taken, resulting in weight gain. There is a clear need to understand the nature of the problem and to use this insight to develop pharmacologic therapies. Developed countries such as Japan and those in Europe are not far behind. A recent study reported that Italy is the leading European nation in weight gain accompanied by an increased prevalence of Type II diabetes in their adolescent population with spread into the childhood group, similar to what is happening in the United States. It appears that the countries with highly-evolved technology centers, penetration of computer-based systems and wide spread cell phone use appear to be moving in this direction. These ''advances'' are each associated with changes such as reduced exercise and increased calorie consumption.

We believe that the PPARs play a central and very important role in the development and treatment of Syndrome X (Desvergne and Wahli, 1999).[8] There are three PPAR isoforms. I will not discuss much here about PPAR alpha (PPARα), which is expressed at high levels in the liver and in the heart, and is involved in beta oxidation of fatty acids. It is the target of a class of prescription drugs called the fibrates, clofibrate and ciprofibrate. PPAR gamma (PPARγ) as we have heard in Dr. Kadowaki's talk, is required for the formation of the adipocyte, and it is the target for a class of drugs called thiazolidinediones (TZDs) ''insulin sensitizers''. Sales from TZDs now comprise a US$4 billion market. The first of the insulin sensitizers was developed in Japan but subsequently because of liver problems was removed from the market. However, the TZDs still comprise a widely used class of drugs and there is much interest in its molecular target PPARγ.

Involvement of PPARs in Heart Disease

PPAR delta (PPARδ) is the receptor that we least understand and this is the primary one upon which I will focus. PPARγ and PPARδ are both involved in the formation of heart disease. The heart, just like the rest of the body, is sensitive to a high-fat diet, poor exercise and genetics. The first infiltration of fatty streaks often develops in childhood and continues in adolescence. It can actually develop in the fetus, if the mother eats a very high-fat diet, and the fetal heart develops atherogenic lesions that persist throughout life. A lesion is populated by macrophages which invade the arterial wall. When these macrophages accumulate lipid, they become foamy and are thus called ''foam cells''. Upon building up in the intima of the artery, they initially form a fatty streak which can subsequently progress into atherosclerosis. Among fifty percent of all Americans and in most Western countries, heart attack is the major cause of death. These cardiac macrophages are very rich in PPARγ and PPARδ and both receptors can play a beneficial role in reversing and preventing heart disease, so we think there may be a natural basis to understanding their medical functions.

PPARγ and the Cholesterol Pathway

I will not describe the data that led to the model shown in Fig. 3, as you all know how hard it is to build a genetic pathway (Lee *et al.*, 2003a).[9] But in this pathway, what we show is that low-density lipoproteins

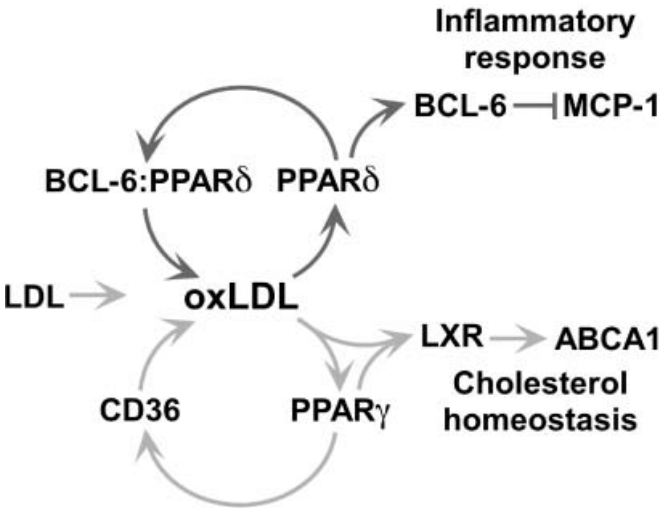

Fig. 3 PPARs are lipid sensors regulating macrophage inflammation and lipid homeostasis.

(LDL), the so-called bad cholesterol, becomes even worse when it becomes oxidized. Macrophages try to clear out oxidized LDL (oxLDL) and as they do, the ligands for PPARγ in this particle activate certain genes such as CD36, a receptor for oxidized LDL. The presence of oxidized LDL creates a cycle that allows the clearance of this toxic lipid particle from the blood into the macrophage. PPARγ also activates another orphan receptor, an oxysterol receptor (LXR), whose lipid ligands are also present in the oxLDL particle. LXR when activated in turn triggers the reverse cholesterol transporter ABCA1, causing the release of HDL from the macrophage. The net result is that the bad cholesterol is processed in the macrophage and released as good cholesterol. Thus the macrophage functions as a living machine that tries to purge the body of bad cholesterol and produce good cholesterol. But if you have too much oxidized LDL, the body is unable to keep up and stores most of it. Through pharmacology, we can try to stimulate the export arm, using chemicals to accelerate HDL release and clear out the artery. That model is currently being tested in patients. So we believe that PPARγ and LXR will play central roles in the future of the treatment of coronary artery disease by non-invasive methods through an orally-active pill.

PPARδ and Clearance of Excess Cholesterol

What about PPARδ? PPARδ does not respond to LDL, but it does respond to VLDL and is a VLDL sensor (Lee *et al.*, 2003b).[6] What is VLDL? VLDL stands for very low density lipoprotein, which is produced by the liver and is a triglyceride-rich particle. We take in dietary cholesterol and other types of fats and fatty acids which are packaged chylomicrons and delivered to the liver where they are repackaged to form LDL and VLDL. These particles comprise the forward arm of cholesterol transport and this is how lipids are delivered through the circulation, to the cells in the body. As triglycerides are stripped from VLDL, it becomes converted to the cholesterol-rich particle, LDL. Excess cholesterol is effluxed back to the liver in the form of HDL. This is the reverse arm of cholesterol transport and is often considered a measure of good cholesterol. In the liver, cholesterol is metabolized into bile acids (BAs) and delivered to the intestine for excretion. While most ($> 95\%$) of all bile acid is resorbed, a small amount ($< 5\%$) is lost in the stool. Because BAs circulate nearly 15 times a day, this is one of the very few mechanisms by which cholesterol can be removed from the body. The problem of overeating cholesterol is that much of it remains in the body, which over a lifetime causes many problems as excess cholesterol is very toxic.

Function of PPARδ in Fat

I will talk about the function of PPARδ in two tissues, adipose and muscle. These are the two key tissues for the metabolism of fats. When we think about metabolism, there are two components that are relevant: fat storage and fat burning. What are the controls for these processes? The two receptors that we have heard about – PPARγ for fat storage and PPARδ for fat burning – are required for the formation of the adipose cell and for storing fat after the cell is formed. Because they are required both to generate and to maintain the fat cells, they are a major target for drug development.

I will describe a series of experiments on the role PPARδ in fat metabolism that were published earlier this year (Wang *et al.*, 2003).[10] We expressed a form of PPARδ in transgenic mice using a fat-specific promoter called aP-2. This form of PPARδ has a VP-tag on it that activates the receptor without a ligand. In this way, it can be selectively activated in fat cells. It causes a net reduction in the fat pad, compared to the wild-type fat pad. The fat cells form and they accumulate lipid, but what we observed is increased metabolism in the cells that causes fat burning and therefore a reduction in total fat content. This has very important implications for body physiology and I will describe a few examples of what happens in these transgenic mice.

First of all, the mice with the activated PPARδ have greatly reduced serum triglycerides – about a 40% decrease, as well as a 20% decrease in serum free fatty acids. This significant reduction, which is what we try to achieve with drugs, we were able to do genetically. If we put these animals on a high-fat diet – one that corresponds to the Western diet, which is about 30% fat,

their lipid levels increased sharply. However, in the transgenic animals, there is a 55% reduction in triglycerides compared to the wild-type mice, demonstrating that there is resistance to the elevation even though they eat the same amount of fat. The same is true for the free fatty acids. There is a resistance – of about 35% reduction. Cholesterol, on the other hand, is not changed. We believe this is because PPARδ controls the triglyceride component of fat but does not influence cholesterol levels itself. How does it do this? PPARδ is switched on in brown and white adipose tissue, which activates a set of target genes that produce a lean phenotype and a depletion of serum lipids. These animals weigh less than wild-type animals. In the brown adipose tissue of wild-type animals on a high-fat diet, the excess fat is stored in vacuoles. But the transgenic mouse burns the excess fat and the brown adipose tissue is found to have a reduced lipid content.

We also examined what happens in leptin receptor-defective (ob/ob) animals that are genetically prone towards obesity. They get fat by excessive eating, and their fat cells contain an excessive amount of fat. If we give them a drug that is specific for PPARδ, the drug causes increased metabolism and fat burning and normalizes the appearance of the brown adipose tissue. This demonstrates that we have an orally active drug that mimics the effect of the activated transgene by increasing fat burning.

To show what happens in the absence of PPARδ function, we knocked out PPARδ completely in some animals. These mice, because they lack PPARδ, cannot upregulate fat burning. As a result, in a three-week experiment on a high fat diet, they nearly doubled their body weight, which is a tremendous amount of weight gain. In the wild-type animals, upregulation of fat burning occurs during the early phase of the experiment, protecting the animals from acute weight gain. On the other hand, the knockout animals gain weight immediately and quite dramatically.

PPARδ Increases Type I Muscle Fiber

The last topic that I want to mention is the consequence of PPARδ activation in muscle. These results were quite surprising. I am going to use the example of two Olympic runners: Frank Shorter, a long-distance runner who has more of a type I (slow-twitch) muscle fiber and the sprinter Maurice ''Mo'' Green, the only human to have run the 100-meter dash in 9.79 seconds without steroids (Ben Johnson did it with steroids) who have type II (fast-twitch) muscle. At birth, we have a certain ratio of type I to type II muscle. It is a genetically determined ratio for each individual, but as you become obese, you lose type I fibers. If you train by running, you gain more type I fiber, so there is an adaptive switch. These two types of muscle fibers are very different. They have different amounts of mitochondria, different troponins, different amounts of myoglobin and myosins and require different amounts of energy to maintain.

We wanted to ask what happens if we express PPARδ in the muscle (Wang *et al.*, submitted).[11] Would it affect muscle metabolism? To our surprise, the transgenic muscle that expresses PPARδ from a myosin-heavy chain gene promoter was much redder (increased levels of type I muscle) than the wild-type. This was quite clearly observed in the gastrocnemius muscle, which normally has a mixed amount of type I and type II fibers. Thus, the wild-type animal had a typical mixture of type I and II fibers, and the transgenic had the slow-twitch, Frank Shorter type I muscle. This was confirmed by fiber type-specific ATPase staining. There are different types of troponins that are expressed in this muscle that confer on it what we would call a long-distance running phenotype.

Increased Running Endurance in PPARδ Transgenic Mice

An experiment that is rarely done in science is a true gain-of-function experiment. Most of what we do is knock genes out; we lose function, we lose gonadal differentiation or we lose some feature of hippocampal function like LTP or LTD. Rarely do we have experiments where we have a net gain-of-function. If I were to ask, ''How would you improve vision?'' or ''How would you improve motor function?'', can you do that simply by making more retinal cells, simply by making more motor neurons? Those are questions that are very difficult to answer. We now had the opportunity to ask the question, ''What happens if you make more type I muscle fiber?'' In this genetically-engineered strain of mice, the question was, do these new slow-twitch fibers become innervated normally by the motor neurons and do they have the right type of response to affect the physiology of these animals?

We addressed this question by putting the mice on a treadmill to see whether the engineered mouse could actually run longer than its wild-type littermates. We placed both wild-type and transgenic mice on treadmills, and monitored various parameters. A platform was situated at the back of the treadmill where the mice, after running to exhaustion, will be deposited. When both hind and fore paws are on the platform, the experiment is terminated.

The wild-type animals were able to run for a little less than 90 minutes, and the transgenics ran for about 150 minutes, about 60 minutes longer, which represented a tremendous increase in time on the treadmill. Since we increased the speed of the treadmill over time,

the transgenics actually ran about twice the distance of the controls. From this we calculated that normal animals could run for about 900 meters before they were exhausted, whereas the transgenics could continue for over 1.8 kilometers, a dramatic difference. This result was very exciting because it indicates that we have found the first transcriptional pathway to alter muscle fiber-type formation in an animal that has a true functional consequence. We believe that this has many consequences besides just running ability. For example, these transgenic animals are apparently resistant to a high-fat diet; although they put on some weight on a high-fat diet, it is nowhere near the amount that the wild-type animals gain. Over the same period of time, the wild-type mice gained almost 20 grams more than the transgenics fed the same diet. In addition, the wild-type animals' fat cells were greatly enlarged relative to the transgenics.

We showed that we can mimic this effect with a drug. We can take wild-type mice and give them a PPARδ-specific drug, conferring on them resistance to weight gain. We thus have an orally active molecule that can prevent weight gain on a high-fat diet. What is the mechanism behind this? We looked at the oxygen consumption and respiration of these animals. We put them into metabolic cages and found that at every time point, the animals treated with the drug had an increased respiratory coefficient. What this means is that the drug causes increased metabolic activity and as result, increased fat burning.

Concluding Remarks

I wish to conclude the presentation by saying that we have two very interesting receptors, PPARγ and PPARδ, that control distinct genetic pathways. We believe that activation of both of these will improve Syndrome X. PPARγ is a known drug target for insulin sensitizing drugs such as Actos and Avandia. We are currently developing drugs for PPARδ, and there will likely be announcements of the first clinical trials of these drugs in humans soon. If the results of these drugs in people are similar to what we have seen in mice, they should produce resistance to weight gain, a lowering of triglycerides and possibly increased insulin sensitivity.

We therefore have a PPAR-mediated metabolic switch that is controlled in part by diet, in part by circadian rhythm, in part by exercise and by certain classes of drugs. It involves two receptors, PPARγ and PPARδ, that activate a set of target genes. Interestingly, although they are expressed in the same cells, they activate different target genes, although a few, like ADRP, a lipid storage gene, are the same. Most of the target genes are very different between these two closely related receptors. Nature appears to have used similarity in receptor structure to control two different, opposing pathways in terms of lipid metabolism – one controlling energy storage, the other controlling energy expenditure. I am very excited about the potential of drugs for these receptors.

This work was done by members of my lab and I want to thank three of the key people who did most of the work I talked about today, Yongxu Wang, Chi-Hao Lee and Ruth Yu. Some of it started with Ajay Chawla who is now at Stanford, Heonjoong Kang, a former chemist in my lab who produces the chemical compounds that we use for these studies. I thank all of my former students in the context of the award, my postdocs, technicians, staff and collaborators for all of their help that helped lead to the Keio Medical Prize Symposium today. Thank you all very much for being here.

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