# The Modification of Biocellular Chemical Reactions by Environmental Physicochemicals

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Environmental risk factors affect human biological system to different extent from modification of biochemical reaction to cellular catastrophe. There are considerable public concerns about electromagnetic fields and endocrine disruptors. Their risk assessments have not been fully achieved because of their scientific uncertainty: electromagnetic fields just modify the bioreaction in the restricted cells and endocrine disruptors are quite unique in that their expression is dependent on the exposure periods throughout a life. Thus, we here describe their molecular characterization to establish the new risk assessments for environmental physicochemicals.

## §1. Environmental risk against health

Our health has been affected by many environmental risk factors, which are dramatically increasing in a parallel to the development of our society. Availability of man-made physicochemicals has created luxury lives, standing by the risks in them.

The current methodology for risk assessment is focused on its source, route, and the amount of those factors exposed. The risk assessment process is divided into four distinct steps: hazard identification, exposure assessment, exposure-response assessment and risk characterization.

The purpose of hazard identification is to evaluate qualitatively the weight of evidence for adverse effects in humans based on the assessment of all the available data on toxicology and modes of action. Exposure assessment is the determination of the nature and extent of exposure to the environmental factors. Exposure-response assessment is the process of quantitatively characterizing the relationship between the exposure received and the occurrence of an effect. Risk characterization is the final step in the risk assessment process. Its purpose is to support risk managers by providing the essential scientific evidence and rationale about risk that they need for decision-making. In risk characterization, estimates of the risk to human health under relevant exposure scenarios are provided.

Risk can be defined as the probability of an adverse effect in an organism, system or subpopulation caused under specified circumstances by exposure to an environmental factor. Risk has three characteristics variables: the type, magnitude and probability of the hazard. In quantitative terms, risks are often expressed in terms of probability estimates ranging from zero to one. A distinction is made between environmental factors with and without threshold levels. In the case of chemicals without threshold levels, many carcinogens, often a linear relationship is assumed be-

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tween exposure (dose) and effects (incidence of cancer). Not all environmental factors present non-threshold cancer risks, but they may affect developmental, reproductive, neurobehavioral, and other body functions. Such effects are often associated with a threshold level and a non-linear S-shaped relationship between dose and effects.

However, scientific uncertainties in the research field of electromagnetic fields and endocrine disrupters make it difficult to assess them. Epidemiologic studies of magnetic fields have consistently shown associations with childhood leukemia, but lack of a known mechanism at such low energy levels and negative animal data suggest that the association is not causal. As to an endocrine disrupter, 'low doses' endocrine disruptors issue still remains unsolved. The low doses define doses below those used in traditional toxicological studies conducted for risk assessment purposes: for bisphenol A, an endocrine disruptor, it is dose below 50 mg/kg body weight/day.

Furthermore, the methodology of the risk assessment currently used is based on the health of adults. Recent research has revealed that children health is much vulnerable to physicochemicals in the environment than those in adulthood. Childhood leukaemia by electromagnetic fields exposure and neurodevelopmental disorders by endocrine disruptors have been attracted particular attention. Thus, it is now necessary to establish the new methodology of risk assessment based on children health.

To first assess the health risk, it is important to estimate the human exposure levels. Extrapolation from experimental animals to humans has been performed under the environmental exposure scenarios of daily life, since it is extremely hard to achieve to estimate the human exposure. For detection of any effects of environmental factors, a biomonitoring system with high sensitivity has been required to be established. To this end, we have to know how environmental factors would affect our health at molecular levels.

Molecular toxicology has revealed that several environmental toxicants induce apoptosis of their target cells. The discovery of apoptogenic nature of environmental toxicants has dramatically changed our view on the toxicological action of the toxicants since it has been long time to believe that their action is necrotic. Thus, it has been expected to develop new means of health risk assessment based on apoptotic signaling. One of advantages is that apoptosis induced by environmental factors occurs before they induce necrosis, indicating that it will make the detection of health effects of the factors earlier than the methods based on necrosis. Another advantage is that apoptotic phenomena are seen by a variety of environmental factors such as cadmium, methylmercury, diesel exhaust particles, uv, dioxin, bisphenol A, and several kinds of phthalates, suggesting that unidentified factors in the environment would be detectable by apoptotic signaling if an apoptosis signal is in general for toxic actions of environmental factors. Ultimately, apoptotic signals leads to cell death, probably due to shut down cellular metabolism in cells affected by the environmental factor before killing all healthy cells left.

## §2. Modification by electromagnetic fields of biochemical reactions *in vitro*

Recent efforts have been made to detect the subtle influences of environmental factors in human biological system without a catastrophe of cell death. Since environmental factors evoke both death signaling and cellular protective reactions against toxicity, cell fate is committed by the resultant which reaction exceeds the others. This escapes the detection of trace effects of toxicants. It has applied to seek the biological effects of electromagnetic fields exposure, leading to a finding of the modification of hormonal melatonin signaling and gene expression of several oncogenes.

Wertheimer and Leeper reported a nearly threefold increase of breast cancer risk among women younger than 55 who lived near power lines, suggesting that electromagnetic fields exposure had accelerated development and growth of breast cancer. Furthermore, increased breast cancer risks were reported in both women and men.

It is not known which organs are primarily involved in sensing electromagnetic fields and their changes. Electrophysiological studies have shown that some of the intrinsic cells of the pineal gland may experimentally be affected by an earth-strength electromagnetic fields and that these cells respond with a depression of their electrical activity. Concerning the mechanism of 'magnetosensitivity', one can assume that an electromagnetic field has a direct effect upon the pineal gland because of the electric current induced inside the body.

Stevens hypothesized that electromagnetic fields can affect pineal gland melatonin secretion *in vivo*, which, in turn, can influence mammary (breast) carcinogenesis. Since then, a number of experimental studies have been conducted in order to test this hypothesis. Kato et al. reported that exposure of albino (Wistar-King) rats for 6 weeks to 50-Hz and 1 $\mu$T suppressed melatonin concentrations, both during the day and during the night, in both plasma and the pineal gland. Olcese and Reuss investigated effects of combined 60-Hz vertical electric field and 60-Hz horizontal electromagnetic fields exposure for 6 weeks on non-human primates and found no signs of a reduction in serum melatonin concentrations in a series of three experiments. However, in another small experiment using a different exposure paradigm, they reported nearly complete suppression of the normal nocturnal rise in serum melatonin concentrations, indicating that different animal species respond differentially to different parameters of time-varying magnetic fields.

In *in vitro* studies, Blask and colleagues demonstrated that melatonin at physiological levels inhibits MCF-7 human breast cancer cell growth. Using MCF-7 cells obtained from Blask, Liburdy reported that electromagnetic fields inhibited the antiproliferlative effects of the hormone, allowing the cancer cells to grow in the presence of melatonin. Furthermore, he revealed the first plausible biological mechanism linking electromagnetic fields exposure to calcium signaling, a fundamental cell process governing many important cellular functions. However, the '$Ca^{2+}$' theory is now subject to debate.

There are many MCF-7 subclones that respond to different degrees of electro-

magnetic fields as well as to estrogen and melatonin. For example, the results that Liburdy and his collaborators obtained have not been reproduced with the MCF-7 cells supplied by the American Type Culture Collection (Manassas, VA). Thus, the MCF-7 cells that have 'magnetosensitivity' (designated 'MF-sensitive MCF-7 cells' in this study) are useful in elucidating the molecular basis of the biological effects of electromagnetic fields.

Therefore, we used MF-sensitive MCF-7 cells provided by Dr. Liburdy (UCLA, Berkley) in order to reveal the molecular mechanism of the biological effects of electromagnetic fields.

In order to expose the cells to magnetic fields, a 50 Hz sinusoidal electromagnetic field was generated in a mu-metal chamber with four Merritt-coil devices. The mu-metal chamber was a cube that was constructed of nickel (80%) and trace metals. The chamber had four ventilation holes (2.54 cm in diameter) on the top and bottom. A temperature probe was placed inside the chamber to monitor temperature continuously. The anti-parallel mode of operation generated opposing magnetic fields that cancelled and resulted in a true sham exposure. When a current was applied to the parallel configuration, a magnetic field was established. Two identical exposure systems were employed in this study. Each coil system was driven by identical signal generators obtained from NF Electronic Instruments Corp. (Yokohama, Japan).

Only 1a melatonin receptors were identified by the [$^{125}$I]-melatonin binding assay and reverse transcription polymerase chain reaction analysis. The molecular cloning of melatonin 1a receptor revealed that it belongs to G protein-coupled receptor superfamily and that it couples to the inhibitory cAMP transduction pathway. Thus, we investigated the effects of electromagnetic fields on these components of the melatonin signaling. Exposures to electromagnetic fields of 100 $\mu$T for 3, 5, and 7 days blocked the melatonin-induced inhibition of cAMP accumulation in a time-dependent manner, while none of the melatonin receptor functions or GTPase and adenylyl cyclase activities were affected. Estrogen-evoked cell proliferation was not altered by electromagnetic fields either. Exposure to 1.2 $\mu$T electromagnetic fields exerted the same effects on the melatonin-signaling pathway as that to 100 $\mu$T. Thus, this is the first study to provide evidence that electromagnetic fields may cause uncoupling in the signal transduction from melatonin receptors to adenylyl cyclase.

We also investigated whether electromagnetic fields exerted the effects on the gene expression in the MCF-7 cells using human glass microarray (Clonthech). There were 1,081 genes on Atlas glass Human 1.0 Microarray. The gene expression levels were calculated as the ratio of the control cells. The levels of gene expression of 95% genes were unchanged. There were 39 genes in which the expression level was increased more than 2-fold, whereas the expression levels of 12 genes were decreased less than 2-fold by electromagnetic fields exposure (1.2 $\mu$T for 1 week). The similar patterns of gene expression were obtained by exposure of 100 $\mu$T electromagnetic fields for 1 week. The extents of the expression were also similar. The highest was about 6-fold.

Thus, exposure of MCF-7 cells to electromagnetic fields modified both protein levels and gene expression levels. It will be further examined if the long-term effects

of these biological modifications would be linked to the disease such as carcinogenesis.

## §3. Neurodevelopmental disorder by endocrine disruptors *in vivo*

The etiology of multifactorial disorders such as neurodevelopmental disorders of attention-deficit hyperactivity disorder (ADHD) or autism has been considered to be the interaction between a range of intrinsic (gene) and extrinsic (environment) factors.

Autism is characterized by language impairments, social deficits, and repetitive behaviors. It occurs far more commonly in males and has an overall incidence of ~1 in 150 births. Monozygotic twins show >70% concordance, higher with broader diagnostic criteria, and much higher than observed in dizygotic twins, strongly suggesting that autism is genetically determined. Children with affected siblings have a higher risk than the general population, suggesting that autism can be inherited at least partially from preexisting genetic variants in parents. Autism is likely to involve many genes. Linkage studies find no single locus of major effect but rather a very minor increase in allele sharing over the entire genome among concordant sibs. Cytogenetic studies, and more recently copy number analyses, support the idea that many loci may contribute the disease.

The symptoms of ADHD are also inattention, excess impulsivity, uncontrolled hyperactivity, and the deficit in social communication, affecting ~5% of children and adolescents and ~3% of adults. ADHD is more frequently diagnosed in boys, with male: female ratios between 3:1 and 4:1. ADHD is defined by the presence of six or more symptoms of inattention and/or hyperactivity-impulsivity that lead to significant impairment in at least two settings and have their onset by age 7 years. Comorbidity is common in ADHD, with oppositional disorders, mood disorders, anxiety, and learning disabilities being the most prevalent conditions, likely reflecting the complexity of the biological etiology. ADHD has a significant genetic component. Data from clinical studies consistently support the familial nature of ADHD. Twin studies of categorically defined ADHD and/or continuous rating scales of hyperactivity, impulsivity, and inattention lead to estimates of heritability of ~60%–90% and reported sibling relative risk ratios of 4.0–8.0

Thus, both disorders seem to be inheritable neurodevelopmental disorders. However, recent evidence in animal models has shown that environmental chemicals *per se*, including endocrine-disrupting chemicals and a pesticide rotenone, cause hyperactivity, seen in patients with ADHD or autism. There has been reports that environmental chemicals cause neurodevelopmental disorders in human: polychlorinated biphenyls (PCBs) distributed in the environment cross the placenta to cause *in utero* injury to the developing brain, correlating with a decrease in intelligence quotient. Second, many chemicals such as dioxins, PCBs, bisphenol A, and heavy metals have been detected in human umbilical cords and cord serum, suggesting that they transfer transplacentally from mother to fetus and that the chemicals might exert their effects on the developing brain. These circumstances made us hypothesize that environmental chemicals including endocrine disruptors might contribute to the incidence of neurodevelopmental disorders such as ADHD and autism.

The pioneer work of the animal model for hyperactivity was carried out by Shaywitz et al. (1976), who demonstrated that rat pups treated with 6-hydroxydopamine via intracisternal administration at 5 days of age developed increased motor activity, leading to cognitive difficulties in shuttle-box learning between 2–4 weeks of age. These observations were strikingly similar to the clinical syndrome of minimal brain dysfunction, called ADHD at present, found in children. In 6-hydroxydopamine-treated rat pups, brain dopamine was depleted, suggesting that brain dopamine may be involved in the pathogenesis of the disorder.

Following the protocol by Shaywitz, we first intracisternally administered an endocrine disruptor into the 5-day-old rat. At 4–5 weeks of age, their spontaneous motor activities were measured by Supermex system (Muromachi kikai Co. Tokyo). In this system a sensor detects and measures the radiated body heat of an animal. A Supermex sensor head consists of paired infrared pyroelectric detectors. This system detects any object with a temperature at least 5°C higher than background within a cone-shaped area with a 6 m diameter and a 110°Cvertex. The sensor monitors motion in multiple zones of the cage through an array of Fresnel lenses placed above the cage and movement of the animal in the $X$, $Y$, and $Z$-axis can be determined. Activity was measured in 15 min increments for 22–24 h and maintained on a 12h light:dark cycle.

Nineteen endocrine disruptors were tested for rat hyperactivity: five phenols, eight phthalates, and six others. 6-Hydroxydopamine was also employed for a positive experimental control. To compare the effects of each endocrine disruptor, they were administered at a fixed amount of 87 nmol: for example, 87 nmol is equivalent to 20 $\mu$g of bisphenol A, 18 $\mu$g p-n-octylphenol, or 29 $\mu$g dicyclohexylphthalate. The extent of spontaneous motor activity exerted by endocrine disruptors was about 1.3$\sim$1.6 fold, compared with that of a control rat. 6-Hydroxydopamine increased their spontaneous motor activity 1.8 fold. Dose dependency was seen in the cases of bisphenol A and dicyclohexyphthalate. Two hundreds nanogramme of endocrine disruptors in the brain was sufficient to cause rat hyperactivity as assessed by the Supermex system used. It was notable that the hyperactivity of the bisphenol A-treated rats in the novel environment was most prominent during nighttime, probably because the chemical-treated rats might be less able to adapt to new environment. The similar pattern was also seen in the dopamine D3 deficient-hyperactive mice, and in dopamine transporter deficient-hyperactive mice.

The reference dose (RfD) of bisphenol A is calculated by the US Environmental Protection Agency as 50 $\mu$g/kg/day by dividing the Lowest-Observed Adverse-Effect-Level (LOAEL) by an uncertainty factor of 1,000. No-Observed –Adverse-Effect-Level (NOAEL) in reproductive toxicity was reported as 50 mg/kg/day, confirming the safety of the RfD.

Therefore, for neural risk assessment of endocrine disruptors, we then evaluated the exposure route of chemicals. Bisphenol A (12$\sim$60 mg/kg) was orally administered into the male Wistar rat aged 5 days–3 weeks. At 4$\sim$5 weeks of age, their spontaneous motor activities were measured by the Supermex system. It was about 1.3 times as active in the nocturnal phase as were vehicle-treated control rats. The long term effects of the chemical resulted in a large reduction of immunoreactivity

for tyrosine hydroxylase, a rate-limiting enzyme for catecholamine synthesis, at 7 weeks of age, where terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive neural cell death were detected in the substantia nigra pars compacta. Immunostaining for glutamic acid decarboxylase, which is involved in $\gamma$-aminobutyric acid synthesis, revealed the immunoreactive enzyme in the substantia nigra pars reticulata, indicating that the effects of bisphenol A are specific to the dopaminergic neurons.

Impairment of immunoreactivity for tyrosine hydroxylase was associated with these hyperkinesias, indicating that the developmental deficit of dopaminergic tone may underlie motor hyperactivity. It has been demonstrated that bisphenol A was converted to bisphenol o-quinone *in vitro*. Tyrosine hydroxylase is an oxidatively labile enzyme whose level of activity is determined, in part, by redox regulation of disulfide linkage with GSH. Catechol-quinone reduced tyrosine hydroxylase activity to an extent that is related to cysteine modifications. Therefore, it is possible that the toxicity of bisphenol A may be attributed to the degeneration of motor neurons leading to hyperkinetics of the rats as seen in the case of 6-hydroxydopamine.

Gene manipulation studies have developed many models of animal hyperactivity. Particularly, the mice lacking the genes for dopamine type 3 receptor or the dopamine transporter were hyperactive. On the other hand, the mice lacking the genes for dopamine type 1, 2, or 4 types receptors show hypoactivity. Locomotor hyperactivity has been associated with not only hypodopaminergic but also hyperdopaminergic animal models, indicating that imbalance in dopamine systems can produce behavioral and cognitive dysregulation.

Furthermore, analysis by reverse transcription polymerase chain reaction revealed that the chemical decreased the gene expression of dopamine transporter in the midbrain. These observations suggest the etiology of rat hyperkinesias might be explained by multiple-hit hypothesis in dopaminergic neurons. The brain may readily be able to compensate for the effects of an individual chemical alone acting on a particular system of the brain. However, when multiple target or functional sites within that particular system are attacked by different mechanisms, the system may no longer be able to homeostatically reregulate itself, thereby leading to sustained or cumulative damage of the dopaminergic neurons.

Hyperactivity among children was first described by von Economo (1931) in cases of encephalic lethergica. Hyperactivity, sleep disorders and antisocial personality disorder are all associated with this disease in childhood and Parkinsonism was observed in adult cases. This suggests that the etiology of hyperactivity in children could involve the potentially irreversible degeneration of dopaminergic neurons since Parkinson's disease is caused by the selective loss of dopaminergic neurons.

Currently, hyperactivity is associated with ADHD or autism, as mentioned as above. The etiology seems to be, in part, associated with dopaminergic tone. Dopamine transporter may be involved in the pathogenesis, as methylphenidate, which increases the synaptic concentration of dopamine by blocking the dopamine transporters, has been used for the treatment of these disorders. Genetic studies have reported the association of certain alleles of the human dopamine type 4 receptor gene or dopamine transporter gene with occurrence of ADHD.

## §4. The expanded Barker's hypothesis

Sporadic neurodegenerative diseases and mental disorders have well-documented environmental causes. Through detailed reconstructions of neonatal and medical histories of birth cohorts in the United Kingdom, David Barker proposed the concept that parameters of fetal, infant, and childhood growth may be predictors of disease in later life. Barker found that infants with low birth weight, small head circumference, and low ponderal index at birth are at increased risk of developing coronary heart disease, hypertension, stroke, insulin resistance, and diabetes as adults. He found also that reduced fetal growth and impaired development during infancy were associated with increased mortality from cardiovascular disease in both men and women, independently of social class and other confounders such as smoking, alcohol consumption, and obesity. Thus, Barker hypothesized that fetal undernutrition during critical periods of vulnerability in early development leads to persistent changes in hormone levels and altered tissue sensitivity to these hormones, permanently altering the metabolism and body structure.

The plausibility of extending the Barker hypothesis to encompass brain development and to explore the impacts of toxic chemicals on brain development was argued. The expanded Barker's hypothesis proposed that the environmental origins of these disorders in later life might be early in life during windows of developmental vulnerability. The vulnerability to environmental factors is dependent on the period of their exposure: in utero and in early postnatal life may be most sensitive. Furthermore, early exposure to environmental toxicants could lead to persistent changes in later life. Thus, children are victim of environmental violence and predictors of disease in later life.

Parkinson's disease is a progressive neurodegenerative movement disorder that is estimated to affect approximately 1% of the population older than 65 years of age. Clinically, most patients present with the cardinal symptoms of bradykinesia, resting tremor, rigidity, and postural instability. A number of patients also suffer from autonomic, cognitive, and psychiatric disturbances. The major symptoms of Parkinson's disease result from the profound and selective loss of dopaminergic neurons in the substantia nigra pars compacta, but there is widespread neuropathology with substantia nigra pars compacta becoming involved later toward the middle stages of the disease. The pathological hallmarks of Parkinson's disease are round eosinphilic intracytoplasmic proteinaceous inclusions termed Lewy bodies and dystrophic neuritis present in surviving neurons. Clinicians noted that patients with Parkinson's disease often had an affected relative. Further studies corroborated these suspicions with the identification and characterization of families that inherited Parkinson's disease in a Mendelian fashion. In contrast, a number of studies utilizing twin registries demonstrated a low rate of concordance in monozygotic and dizygotic twins, indicative of a lack of genetic susceptibility in Parkinson's disease. Recent studies identified the loci responsible for rare Mendelian forms of Parkinson's disease.

Despite genetic advances, much research has continued to focus on the contribution of nongenetic or environmental factors to the development of Parkinson's disease. Parkinson's disease is primarily a sporadic disorder and its specific etiology

is incompletely understood. The environmental origins of human sporadic Parkinson's disease might be also early in life. Possible explanation for this mechanism is that early exposures to neurotoxic chemicals reduce the number of dopaminergic neurons in critical areas of the brain such as the substantia nigra to levels below those needed to sustain function in the face of the neuronal attrition associated with advancing age.

An animal model of Parkinson's disease has been developed by selective dopaminergic degeneration with neurotoxicants, pesticides, or endocrine-disrupting chemicals in adult animals. Although one of etiologies of animal models of both Parkinson's disease and hyperkinetic disorders is apparently dopaminergic dysfunction, behavioral features of both diseases are opposite, e.g. hypokinesia versus hyperkinesia. This fact suggests that the same action of an environmental toxin would develop very different disorders, dependently of periods of exposure.

## §5. Remarks

Human biological system consists of not only physicochemical but also psychiatric networks. As seen above, environmental factors affect both dimensions to different extent from modifications of biochemical reaction to cellular catastrophe. Future studies in understanding the affection of environmental risk factors on the body and mind, and our adaptation are possible approach to look for an answer to '*what is life?*'

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