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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer; Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonweal on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with "low doses" of BPA and many of theses negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the "low dose" animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of "low dose" of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies "low doses" involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg^{-1} day^{-1}) in studies with rats and mice. The 50 mg (kg^{-1} day^{-1}) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 μg (kg^{-1} day^{-1}). The current reference dose is thus based on "high dose" experiments conducted in the 1980s [7]. (2) "Low dose" also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the "low dose" range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first "low dose" BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of "high concern" only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

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system in female rats and thus to be an "environmental estrogen" in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a "weak" estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

2. Purpose and organization of the BPA meeting

2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel's report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*.The following specific concerns about BPA led to the five expert panels being established:

(1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
(2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
(3) BPA causes a wide range of adverse effects at "low doses" that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
(4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.
(5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
Panel (3) *In vivo* studies of BPA at "low doses" in laboratory animals.
Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

(1) At least two members from each of the five panels.
(2) A person from each panel who had published on BPA.
(3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
(4) A person with experience in the process of reaching consensus.
(5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.

Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).

Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- "We are confident of the following": this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- "We believe the following to be likely but requiring confirmation": This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

4.1. Based on existing data we are confident of the following

4.1.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).
2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

4.1.2. Issue 2: Wildlife—laboratory animal research connection

1. BPA is found in the environment: aquatic, terrestrial and air.
2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: 0.3–4.4 ng ml^{-1} (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml^{-1} (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of 25 μg kg^{-1}, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

4.1.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters "epigenetic programming" of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.
6. Life stage impacts the pharmacokinetics of BPA.

4.2. We believe the following to be likely but require confirmation

4.2.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive subpopulations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes (α/ß; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in the their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.

4. In wildlife and laboratory studies, BPA induces alteration in steroid biosynthesis/ metabolism/excretion.
5. Wildlife residing in sediment is likely exposed to higher levels of BPA.

4.2.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure is likely to be continuous, unlike exposure in most laboratory animal studies of BPA pharmacokinetics.

4.2.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Clearance of BPA in the fetus is reduced compared to other life stages. Different effects and metabolic clearance mechanisms are also observed in neonatal and adult animals. Conjugation (glucuronidation) and other mechanisms of metabolic clearance of BPA thus vary throughout life.
2. Exposure to BPA during different life stages differentially influences reproductive cancer etiology and progression, and exposure during sensitive periods in organogenesis may increase susceptibility to development of cancers in some organs, such as the prostate and mammary glands.
3. Early life exposure to environmentally relevant BPA doses may result in persistent adverse effects in humans.
4. The function of the immune system can be altered following adult exposure to BPA.
5. Effects on insulin metabolism occur following adult exposure.

4.3. Areas of uncertainty and suggestions for future research

4.3.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. Since BPA can act as an agonist or an antagonist in different tissues and against different background physiological states, the specific co-regulators that mediated these different responses of BPA need to be elucidated based on *in vitro* mechanistic studies, which should be confirmed *in vivo*.
2. Research is needed on specific receptor sub-types (i.e., classical nuclear and non-classical membrane-associated estrogen receptors) in relation to the potency of BPA in different tissues.
3. The identification of multiple estrogen receptor genes and variants as well as different co-regulators with different activities reveals that different levels of potency of BPA could be obtained by complex interactions between these different components that would not be predicted in homogeneous recombinant systems.

4.3.2. Issue 2: Wildlife—laboratory animal research connection

1. To directly relate the effects seen in wildlife with BPA exposure, biomonitoring data are needed from wildlife. In addition to BPA levels, these studies should assay total estrogenic and antiandrogenic activity from other contaminants.
2. There is a need to examine sensitive endpoints in wildlife that have been identified in laboratory animals.
3. There are substantial amounts of plastic debris within marine and fresh water ecosystems, and studies are needed to examine the impact of BPA in the environment on aquatic organisms. Doses used in laboratory experiments involving wildlife should reflect environmental exposures.
4. More studies need to be done with BPA in invertebrates, and a fundamental understanding of estrogen action in invertebrates is required.
5. Studies should determine if amplification of BPA through the food chain occurs, particularly under anaerobic or hypoxic conditions due to the lack of microbial or photodegradation.
6. Future research emphasis should be placed on populations of aquatic animals exposed to landfill leachate and sewage effluent, as these are the primary point sources for BPA exposure.

4.3.3. Issue 3: Laboratory animal research—human exposure connection

1. Even though there have been attempts to estimate daily human intake of BPA, these estimates require many assumptions. The best measures we have to estimate whether humans may be affected by current exposures to BPA are levels in blood (not exposure levels), which can be related to blood levels in experimental animals after acute exposures. Known sources of human exposure to BPA do not appear sufficient to explain levels measured in human tissues and fluids.
2. While BPA is not persistent in the environment or in humans, biomonitoring surveys indicate that exposure is continuous. This is problematic because acute animal exposure studies are used to estimate daily human exposure to BPA, and at this time, we are not aware of any studies that have examined BPA pharmacokinetics in animal models following continuous low level exposures. Measurement of BPA levels in serum and other body fluids suggests that either BPA intake is much higher than accounted for, or that BPA can bioaccumulate in some conditions such as pregnancy, or both. Research using both animal models, as well as epidemiology studies, are needed to address these hypotheses, and this research needs to better mimic the apparent continuous exposure of humans to BPA.
3. More comprehensive exposure and biomonitoring studies are needed, especially in developing countries.
4. In both animal and human studies, internal exposure measures need to be related to health effects. In particular, there is a need for epidemiological studies relating health outcomes to BPA exposure, particularly during sensitive periods in development. These studies should be based on hypotheses from findings in experimental animals. This will require additional development of appropriate biomarkers in animal studies that can be used in epidemiological research.

4.3.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. While there is a great need to continue studying prenatal and perinatal exposures in laboratory animal studies, many organs and endpoints continue developing at later stages (throughout puberty and adolescence). Additional studies are needed during these later periods of development.
2. Additional research is needed regarding exposure to BPA in adulthood to determine whether post-exposure effects are temporary or are permanent and associated with subsequent age-related diseases.
3. Because aging adults lose repair mechanisms, metabolic enzymes, and imprinted genes, the possibility that adult exposures (long-term, low level) can increase the risk of cancers and other conditions during aging should be addressed with additional human research and the development of appropriate animal models.
4. Epigenetics should be examined as a potential mechanism mediating developmental effects as well as the trans-generational effects of BPA and other contaminants. Potential effects of adult exposures also need to be examined in relation to disruption of epigenetic changes that occur normally during aging.
5. Trans- and multi-generational effects of BPA must be examined in laboratory animals and humans.
6. There is a need for studies that involve collection of human blood and urine from humans at several life stages, with specific emphasis on infants and young children and continued monitoring throughout adulthood. Additionally, there is a need to characterize the basis for the variability in BPA levels in studies examining both human urine and serum.
7. There is a need for research on the genetic basis for differences in susceptibility to BPA and other contaminants.
8. Studies are needed on comparative BPA pharmacokinetics in invertebrates and vertebrates (non-human primates included).
9. There is a need to measure total endocrine disrupter load in humans and wildlife. Therefore, biomarkers of endocrine disrupter exposure are necessary.
10. There is a need for more research directed at examining human exposure, pharmacokinetics and health effects of selected BPA precursors (i.e., BADGE, BISGMA, and BISDMA) and metabolites (e.g., halogenated BPAs).
11. There is a need for more studies focused on identification of other (non-estrogen-receptor mediated) mechanisms of action of BPA.
12. Effects of chemicals on the immune system are life stage dependent, and identifying the life stage dependency for BPA effects on the immune system is necessary. In addition, studies examining BPA effects on the immune system in wildlife are necessary.

5. Conclusions

The published scientific literature on human and animal exposure to low doses of BPA in relation to *in vitro* mechanistic studies reveals that human exposure to BPA is within the range that is predicted to be biologically active in over 95% of people sampled. The wide range of adverse effects of low doses of BPA in laboratory animals exposed both during development and in adulthood is a great cause for concern with regard to the potential for similar adverse effects in humans. Recent trends in human diseases relate to adverse effects observed in experimental animals exposed to low doses of BPA. Specific examples include: the increase in prostate and breast cancer, uro-genital abnormalities in male babies, a decline in semen quality in men, early onset of puberty in girls, metabolic disorders including insulin resistant (type 2) diabetes and obesity, and neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD).

There is extensive evidence that outcomes may not become apparent until long after BPA exposure during development has occurred. The issue of a very long latency for effects *in utero* to be observed is referred to as the developmental origins of adult health and disease (DOHaD) hypothesis. These developmental effects are irreversible and can occur due to low dose exposure during brief sensitive periods in development, even though no BPA may be detected when the damage or disease is expressed. However, this does not diminish our concern for adult exposure, where many adverse outcomes are observed while exposure is occurring. Concern regarding exposure throughout life is based on evidence that there is chronic, low level exposure of virtually everyone in developed countries to BPA. These findings indicate that acute studies in animals, particularly traditional toxicological studies that only involve the use of high doses of BPA, do not reflect the situation in humans.

The fact that very few epidemiological studies have been conducted to address the issue of the potential for BPA to impact human health is a concern, and more research is clearly needed. This also applies to wildlife, both aquatic and terrestrial. The formulation of hypotheses for the epidemiological and ecological studies can be greatly facilitated by the extensive evidence from laboratory animal studies, particularly when common mechanisms that could plausibly mediate the responses are known to be very similar in the laboratory animal models, wildlife and humans.

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Glycolytic inhibition by mutation of pyruvate kinase gene increases oxidative stress and causes apoptosis of a pyruvate kinase deficient cell line

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Objective. **SLC3 is a Friend erythroleukemic cell line established from the *Pk-1*^{slc} mouse, a mouse model of red blood cell type-pyruvate kinase (R-PK) deficiency. This study was aimed to elucidate the mechanisms attributing to apoptosis induced by R-PK deficiency.**

Materials and Methods. **SLC3 and a control Friend cell line, CBA2, were cultured in a condition of glucose deprivation or supplementation with 2-deoxyglucose, and apoptosis was detected by annexin V. We established two stable transfectants of SLC3 cells with human R-PK cDNA, and examined the effect of R-PK on an apoptotic feature by cell cycle analysis. Intracellular oxidation was measured with 2′,7′-dichlorofluorescin diacetate. DNA microarray analysis was performed to examine gene-expression profiles between the two transfectants and parental SLC3.**

Results. **SLC3 was more susceptible than CBA2 to apoptosis induced by glycolytic inhibition. The forced expression of R-PK significantly decreased cells at the sub G_0/G_1 stage in an expression-level dependent manner. Microarray analysis showed that proapoptotic genes, such as *Bad*, *Bnip3*, and *Bnip3l*, were downregulated in the transfectants. In addition, peroxiredoxin 1 (*Prdx1*) and other antioxidant genes, such as *Cat*, *Txnrd1*, and *Glrx1* were also downregulated. A significant decrease of dichlorofluorescein fluorescence was observed by R-PK expression. Preincubation with a glutathione precursor showed a significant decrease of apoptosis.**

Conclusion. **These results indicated that glycolytic inhibition by R-PK gene mutation augmented oxidative stress in the Friend erythroleukemia cell, leading to activation of hypoxia-inducible factor-1 as well as downstream proapoptotic gene expression. Thus, R-PK plays an important role as an antioxidant during erythroid differentiation.** © **2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Glycolysis is an essential metabolic pathway in all organisms. Pyruvate kinase (PK) is a key glycolytic enzyme, and has four isoenzymes in mammals, designated M_1, M_2, L (liver), and R (red blood cell). In humans, these isozymes are encoded by two structural genes, *PKM* and *PKLR*, respectively [1]. M_2-PK is the only isozyme that is active in early fetal tissues and also almost ubiquitously expressed in adult tissues, including hematopoietic stem cells, progenitors, leukocytes, and platelets. Red blood cell type-pyruvate kinase (R-PK) becomes a major isozyme during erythroid differentiation/maturation [2,3], and in mature red blood cells (RBCs), R-PK is the only detectable PK isozyme. Deficiency of R-PK causes shortened RBC survival, resulting in hemolytic anemia. In humans, PK deficiency is the most prevalent glycolytic enzyme defect, which is responsible for hereditary hemolytic anemia [4,5].

We have previously established SLC3 [6], a line of Friend erythroleukemic cells from the *Pk-1*^{slc} mouse [7], which has chronic hemolytic anemia with marked splenomegaly due to a missense mutation of the murine *Pklr* gene [8]. SLC3 showed spontaneous apoptosis during routine passage and in vitro erythroid differentiation by butyrate exacerbated apoptosis of SLC3 [6]. Recently, we examined the spleen of a subject with severe PK deficiency [9], and discovered enhanced extramedullary hematopoiesis as well as apoptotic erythroid cells. Enhanced apoptosis

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was also identified in TER119-positive erythroid cells isolated from *Pk-1*^{slc} mice [10]. These results provide evidence that the metabolic disturbances in PK deficiency affect not only the survival of RBCs but also the maturation of erythroid progenitors, which results in apoptosis.

In this study, we examined whether Friend erythroleukemic cell lines showed apoptosis when glycolysis was inhibited. To evaluate whether overexpression of the normal R-PK gene ameliorated apoptosis, we established stable transfectants of SLC3 and compared their apoptotic characteristics and transcriptional profiles with parental SLC3. We present here several pieces of evidence, revealing the biological significance of R-PK to suppress oxidative stress during erythroid differentiation.

Materials and methods

Cell culture and flow cytometric analysis

Friend erythroleukemic cell lines SLC3 and CBA2 have been described previously [6]. Both cell lines are maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 20 μM 2-mercaptoethanol, and a mixture of penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA).

To evaluate the adverse effects of glycolytic inhibition, cells were cultured in either glucose-free RPMI-1640 (Invitrogen) or RPMI-1640 with 2-deoxyglucose (2-DG) at final concentrations of 0.1, 1, and 10 mM. Iscove's modified Dulbecco's medium containing 110 mg/L sodium pyruvate, and RPMI-1640 containing no pyruvate.

Flow cytometric analysis was performed by EPICS XL and analyzed with software, EXPO32 ADC (Beckman-Coulter, Fullerton, CA, USA). Annexin V-Alexa568 and rhodamine 123 were obtained from Roche Diagnostics (Basel, Switzerland) and Sigma, respectively. To examine the effect of N-acetyl-L-cysteine upon apoptosis, we preincubated cells in RPMI-1640 supplemented with 10 mM N-acetyl-L-cysteine for 12 hours, followed by 12- to 24-hour incubation with RPMI-1640.

Establishment of stable transfectants expressing normal R-PK in SLC3 cells

We constructed a human R-PK cDNA expression plasmid vector in erythroid cells. A 1.7-kb fragment covering the entire coding region of human R-PK cDNA [11] was introduced into *Kpn*I-*Eco*RV sites of pcDNA3.1 (Invitrogen). Plasmid DNA was purified with an EndoFree Maxi DNA purification kit (Qiagen, Hilden, Germany). Transfection was done with Effectene Transfection Reagent (Qiagen) as indicated by the manufacturer. Transfected cells were selected using G418 (400 μg/mL).

RT-PCR, Western blotting, and enzyme assay

Total cellular RNA was extracted with an RNeasy purification kit (Qiagen), and 2 μg RNA was reverse-transcribed (RT) at 42°C for 90 minutes with 50 pmole oligo (dT)17 primer, 0.5 U/μL cloned RNase inhibitor (Takara Bio, Shiga, Japan), 10 mM dithiothreitol, 1 mM deoxyribonucleoside triphosphate, and 50 U Expand Reverse Transcriptase (Roche Diagnostics). Aliquots (1/10) were subjected to PCR using primer pairs specifically amplified with human and murine R-PK cDNA, hRPK-F (5′-TGGCCCAGC CTACCCTTGTA-3′)/hRPK-R (5′-CTTAAAGGTGGGGCTTTG GA-3′) and mRPK-F (5′-GCAGATGATGTGGACCGAAG-3′)/ mRPK-R (5′-CTAGATGGCAGATGTGGGACTA-3′), respectively. The reaction mixtures were subjected to 40 cycles of amplification consisting of 94°C for 20 seconds, 60°C for 10 seconds, and 72°C for 10 seconds for hRPK and 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds for mRPK in a GeneAmp PCR system 2400 (Roche Diagnostics, Switzerland), and separated using 2% agarose gel electrophoresis.

For Western blot analysis, cells were harvested, followed by washing with phosphate-buffered saline twice. Following three-times freezing and thawing in extraction buffer (10 mM Tris/ HCl, pH 8.0, 10 mM $MgCl_2$, 0.003% 2-mercaptoethanol, 0.02 mM ethylenediamine tetraacetic acid), cell extracts were obtained for Western blot analysis. Protein assays were performed by the method of Bradford using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).Western blot analysis was conducted using anti-rat L-PK (kindly provided Tamio Noguchi, Nagoya University) and ECL advance Western Blotting Detection Kit (Amersham Biosciences, Buckinghamshire, UK).

PK and lactate dehydrogenase (LDH) activity was measured, as described [12].

Microarray analysis

To prepare high-quality total cellular RNA for the GeneChip assay, RNA was extracted with modified protocols using the TRIzol LS (Invitrogen) and RNeasy purification kit (Qiagen). Briefly, cells were harvested with no washing step, and immediately homogenized with the RLT buffer. The lysate was then mixed with 3 volumes of the TRIzol LS. After a 10-minute incubation at room temperature, the sample solution was mixed with an equal volume of chloroform. The sample was centrifuged at 10,000*g* for 15 minutes at 4°C, and then the upper aqueous phase was transferred to a fresh tube. After mixing with an equal volume of 70% ethanol, the sample was incubated for 10 minutes at room temperature. Without any flash step, the sample solution was transferred to the RNeasy column, and then processed by the manufacturer recommended protocol.

To normalize the variation in data based on the cell count, we used *Bacillus subtilis* RNA for an external standard signal, which was added to the cell lysate in proportion to the sample's DNA contents [13]. Ten microliters of cell lysate was provided for DNA quantification using Picogreen (Invitrogen). GeneChip (Affymetrix, Santa Clara, CA, USA) analysis was carried out according to the Affymetrix-recommended protocols. Processed RNA was hybridized to the Affymetrix Murine Genome 430A arrays (22960 probe sets). Signal values were calculated from scanned images by the Affymetrix Microarray Operation System (GCOS). The cell sample was pooled from six culture dishes at each condition and one GeneChip was used per one pooled sample.

Data analysis

Data were normalized by an original program (SCal), which processes data in proportional conversion based on the DNA content of each biosample [13]. This DNA content-based normalization method improves the measurement accuracy of GeneChip. For example, a series of samples was measured by quantitative PCR and Affymetrix GeneChip microarrays using this method, and the results showed up to 90% concordance [13].

To identify differentially expressed genes, we used an empirical threshold calculated by an original algorithm (Fx). The Fx threshold is based on the signal intensity level and is calculated as follows: $Y = X \cdot (1 + RC^{(w\text{-}\log X)})$ and $Y = X \cdot (1 + C^{(w\text{-}\log X)})^{-1}$ (Fx1 and Fx2 respectively; C and w are constant parameters reflecting actual measurement data by GeneChip hybridized with the standard sample). C and w were set to 3.0 and 2.5, respectively, which was equivalent to $p < 0.02$. In the scatter plot, the spots above the Fx1 line were evaluated as upregulated, and the spots below the Fx2 line were evaluated as downregulated.

Results

SLC3 is more susceptible than the control to apoptosis due to glycolytic inactivation

Figure 1 shows flow cytometric analysis using annexin V (horizontal axis) and rhodamine 123 (vertical axis) to examine the effects of glycolysis inhibition on Friend leukemic cells with or without R-PK mutation. SLC3 showed spontaneous apoptosis during routine passage, and apoptosis preceded mitochondrial dysfunction in the R-PK–deficient erythroleukemia cells as reported previously [6]. The result showed that a part of apoptotic cells kept similar mitochondrial transmembrane potentials and that SLC3 were much more susceptible to glucose deprivation as well as 2-DG.

Overexpression of wild-type R-PK decreases apoptosis of SLC3

In order to evaluate how wild-type R-PK rescues apoptotic phenotypes, we established two stable transfectants of SLC3 with overexpression of the human R-PK cDNA. Figure 2 shows RT-PCR and Western blot analysis of a parental SLC3 and SLC3-hRPK.Hi (hRPK.Hi) and SLC3-hRPK.Lo (hRPK.Lo). As shown in Figure 2A, the expression level of the transgene was higher in hRPK.Hi than hRPK.Lo. Overexpression of human R-PK suppressed endogenous R-PK expression as observed in the lane of hRPK.Hi.

Enzymatic analysis of transfectants revealed that PK activities of hRPK.Lo and Hi were 17.2 and 24.2 IU/mg protein, respectively. The PK activity of hRPK.Hi was almost comparable to parental SLC3, 23.5 IU/mg protein. It should be noted that endogenous LDH activity was decreased by transgene expression, leading to a PK/LDH ratio increase from 0.4 (SLC3) to 0.48 (hRPK.Lo) and 0.6 (hRPK.Hi).

We evaluated apoptosis of the two transfectants by cell cycle analysis. Figure 2C shows that the expression of wild-type R-PK decreased the number of cells at the sub-G_0/G_1 stage. While hRPK.Lo showed almost the same number of sub-G_0/G_1 cells (55.5%) as SLC3 (57.4%), only 19.3% of hRPK.Hi were arrested at the sub G_1-stage. Because apoptotic cells were rescued from apoptosis in an R-PK expression level-dependent manner, it is most likely that R-PK activity is required to suppress apoptosis of erythroid cells.

Microarray analysis elucidates the differential expression of genes involved in reactive oxygen species removal, cell cycle, and apoptosis

Gene expression profiles between the two transfectants and the parental SLC3 cell line were analyzed by DNA microarray analysis. After exchanging culture medium, SLC3, hRPK.Lo, and Hi were sampled at 24 and 67 hours, which were the phase of reentry into cell cycling and of subconfluence, respectively. Transgene expression upregulated only about 2% (469 probe sets) of genes, whereas approximately 25% (5754 probe sets) of genes were downregulated both in hRPK.Hi and hRPK.Lo at 24 and/or 67hours. As shown in Figure 3B, major categories of the downregulated genes involved the cell cycle, development, and apoptosis. Proapoptotic genes including *Bad*, *Bnip3*, and *Bnip3l*, as well as *Casp 2, 6, 7,* and *8* were downregulated (Figs. 3A and 4).

Genes of key glycolytic enzymes such as hexokinase-2 (*Hk2*), phosphofructokinase (*Pfkl*), phosphoglycerate kinase (*Pgk1*), and PK (*Pklr*) were downregulated, and expression levels were characteristically decreased after 67 hours of transfection, suggesting that suppression requires protein synthesis.

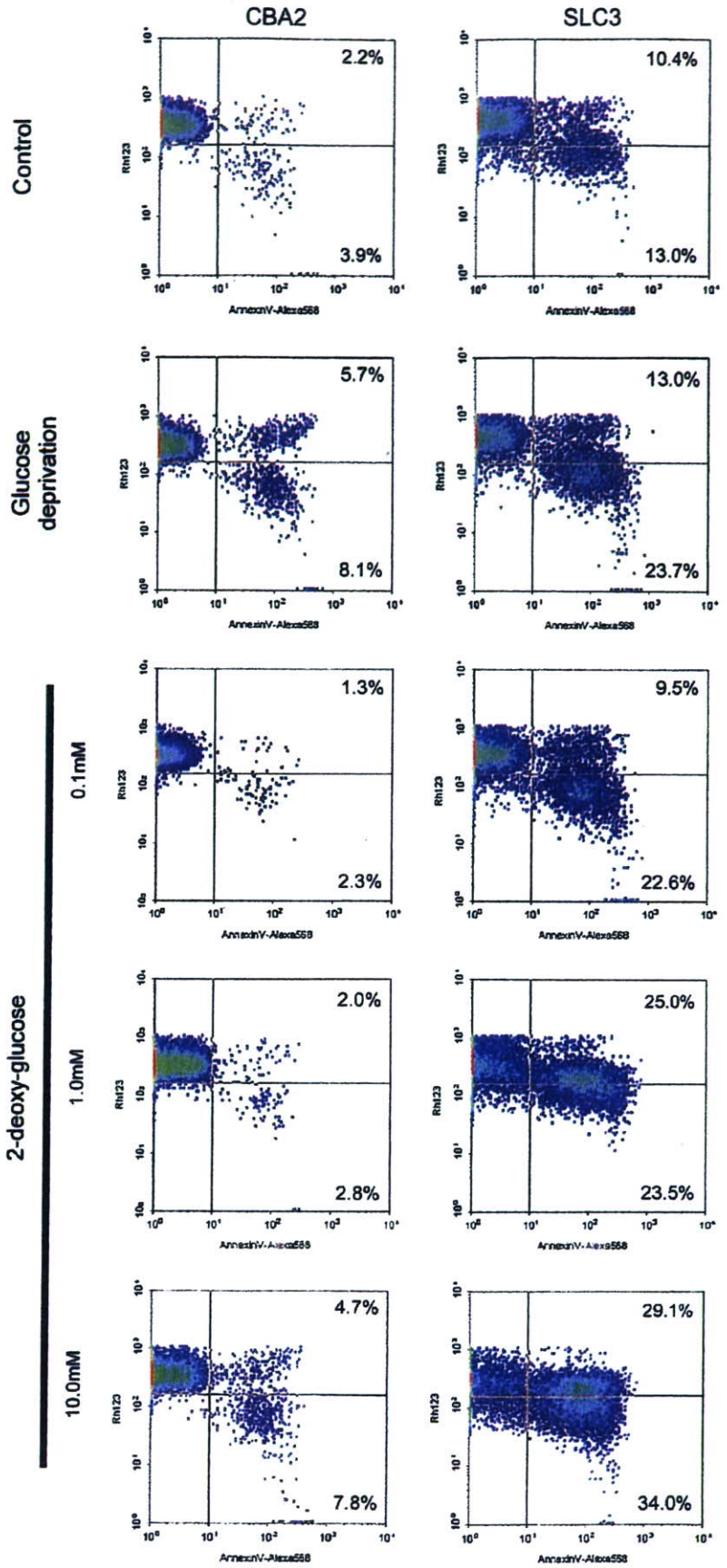
It should be noted that genes for antioxidant protein, such as peroxiredoxin 1 (*Prdx1*) and related genes, such as catalase (*Cat*), thioredoxin reductase 1 (*Txnrd1*), and glutaredoxin 1 (*Glrx1*), which have a role in the modulation of oxidative stress, are also downregulated. As for *Prdx2*, expression change by the transgene was not evident. Intracellular reactive oxygen species (ROS) are known to cause DNA damage, inducing the expression of DNA repair genes. In this experiment, expressions of genes involved in DNA repair were decreased, including *Brca1*, *Brca2*, and *Rad51*.

PK gene mutation and glycolytic inhibition by 2-DG augment intracellular ROS

We examined intracellular ROS in SLC cells and control CBA2 cells by 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), an indicator of the intracellular formation of hydrogen peroxide and free radicals. Nonfluorescent DCFH-DA turns into DCFH (2′,7′-dichlorofluorescin) in the presence of hydrogen peroxide, and then DCFH is quickly photo-oxidized to fluorescent DCF (2′,7′-dichlorofluorescein).

Figure 5A shows that SLC3 is hypersensitive to a glycolytic inhibitor, 2-DG, producing intracellular DCF by adding 1 mM 2-DG. In contrast, control CBA2 cells do not produce DCF even at 10 mM 2-DG for 30 minutes.

Reduced glutathione (GSH) is an important antioxidant in erythrocytes. GSH is produced by a two-step enzymatic reaction involving γ-glutamylcystein synthetase and glutathione synthetase (GSH-S). Apoptosis induced either by the glycolytic gene mutation (SLC3) or the glycolytic inhibitor (CBA with 2-DG) was suppressed by preincubation with the glutathione precursor, NAC (Fig. 5B). Finally, the


CBA2
SLC3
Control
Glucose deprivation
2-deoxy-glucose
0.1mM
1.0mM
10.0mM
2.2%
3.9%
10.4%
13.0%
5.7%
8.1%
13.0%
23.7%
1.3%
2.3%
9.5%
22.6%
2.0%
2.8%
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23.5%
4.7%
7.8%
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34.0%
Rh123
AnnexinV-Alexa568

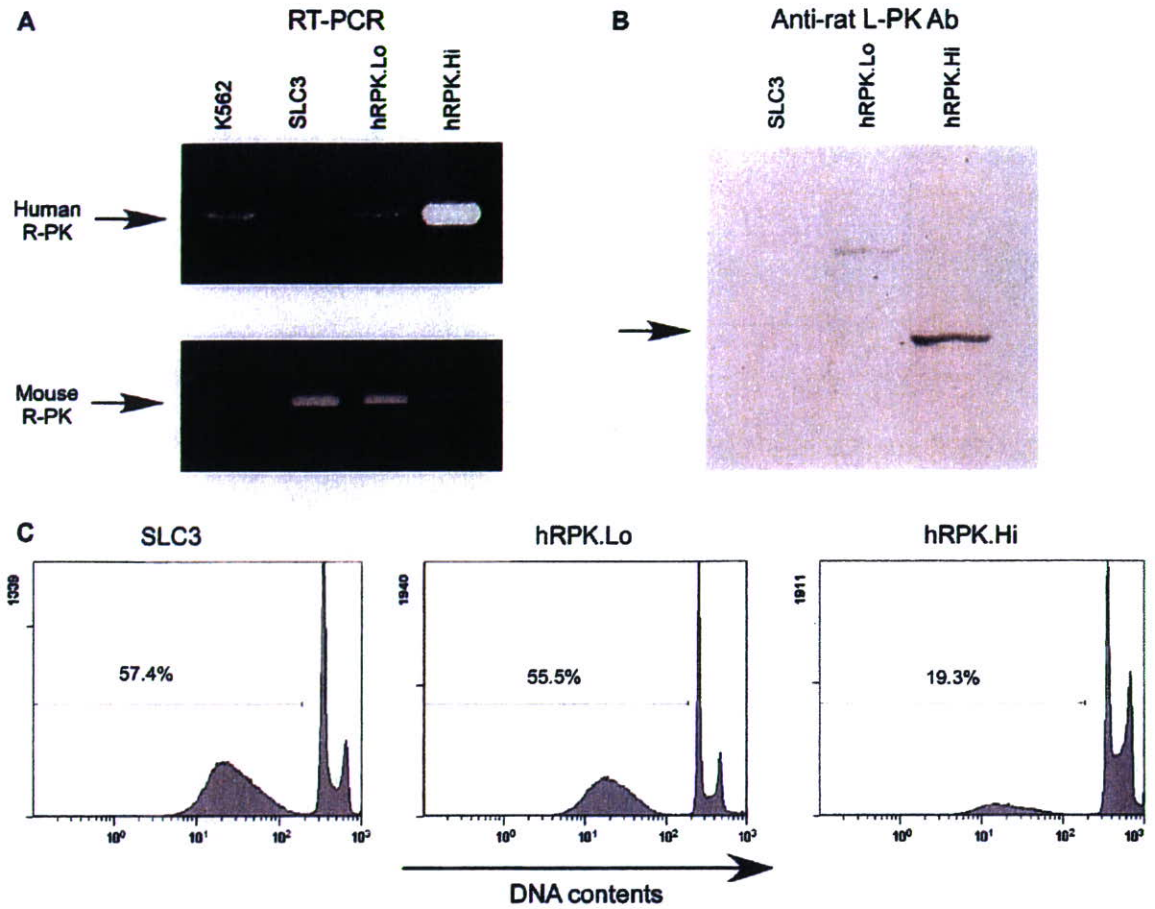


Figure 2. Establishment of the transfectants, SLC3-hRPK.Hi (hRPK.Hi) and SLC3.hRPK.Lo (hRPK.Lo), by introducing the human red blood cell type-pyruvate kinase (R-PK) gene into murine R-PK–deficient cells. Transgene-expression was confirmed by reverse transcriptase polymerase chain reaction (**A**) and Western blotting (**B**). The expression level of hRPK.Hi was higher than that of hRPK.Lo. (**C**) Apoptosis induction in the PK-deficient cells and transfectants. Transfected human R-PK recovered the glycolytic function and showed reduced spontaneous apoptotic changes. The numbers in figures represent the apoptotic change ratio.

forced overexpression of the PK gene reduced intracellular ROS in an expression-level dependent manner (Fig. 5C).

Discussion

Overexpression of human R-PK in SLC3 results in the reduction of apoptotic cells (Fig. 2C), and DNA microarray analysis showed that genes involved in the cell cycle, DNA repair, and antioxidants were downregulated. In general, gene expression levels of transfectants were lower than that of SLC3 (Fig. 3). However, aberrant apoptosis and invalid cell proliferation were restrained in the transfectants. These observations suggested that the cellular activity was not suppressed but was reverted to the normal level by the transgene. It is most likely that the candidate genes suppressed in transfectants were induced in R-PK mutant cells.

Although there were several candidate genes attributing to apoptosis-induction in SLC3, it was still unclear whether these genes were associated with each other or independent. However, there was a possibility that a signal cross-talk phenomenon occurred [14]. *Bad*, a gene encoding a member of the Bcl2-family proapoptotic molecules in mitochondria was significantly downregulated by the transgene (Figs. 3A and 4). Danial et al. [15] reported that *Bad*, BCL2-antagonist of cell death, formed a functional holoenzyme complex together with several molecules, such as glucokinase (hexokinase-4) in liver mitochondria, and contributed to apoptosis induction by glucose deprivation. Our observation suggested that *Bad*

Figure 1. Apoptosis induced by glycolytic inhibition in erythroid cell lines. Glucose deprivation or exposure to 2-deoxyglucose inhibits glycolysis and finally causes apoptosis. The red blood cell type-pyruvate kinase (R-PK)–deficient erythroid cell line (SLC3) is more susceptible than wild-type cells (CBA2) in these conditions. The horizontal axis shows AnnexinV-Arexa568 (= apoptotic change) and the vertical axis shows Rhodamin123 fluorescence (= mitochondrial membrane potential).

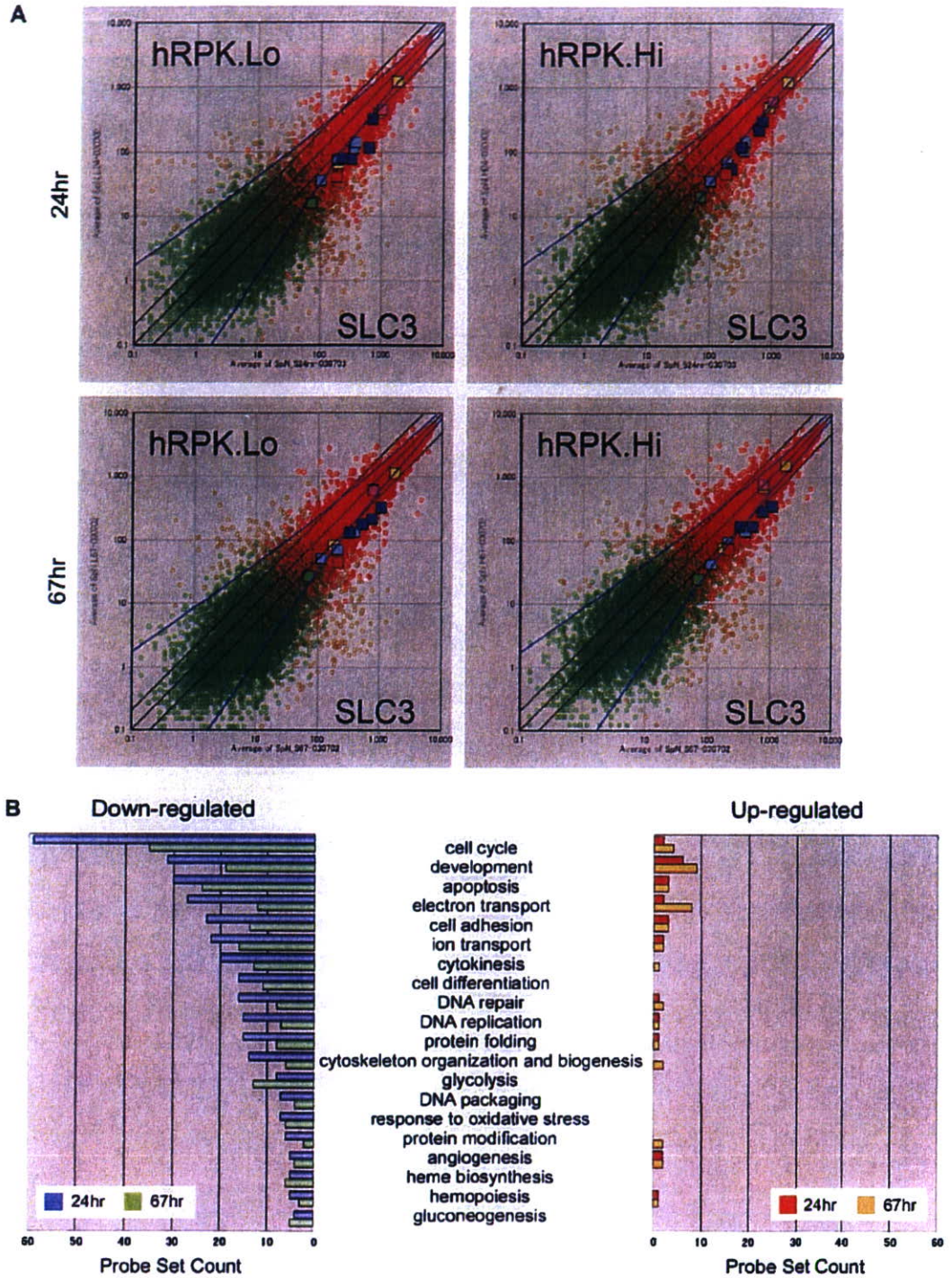
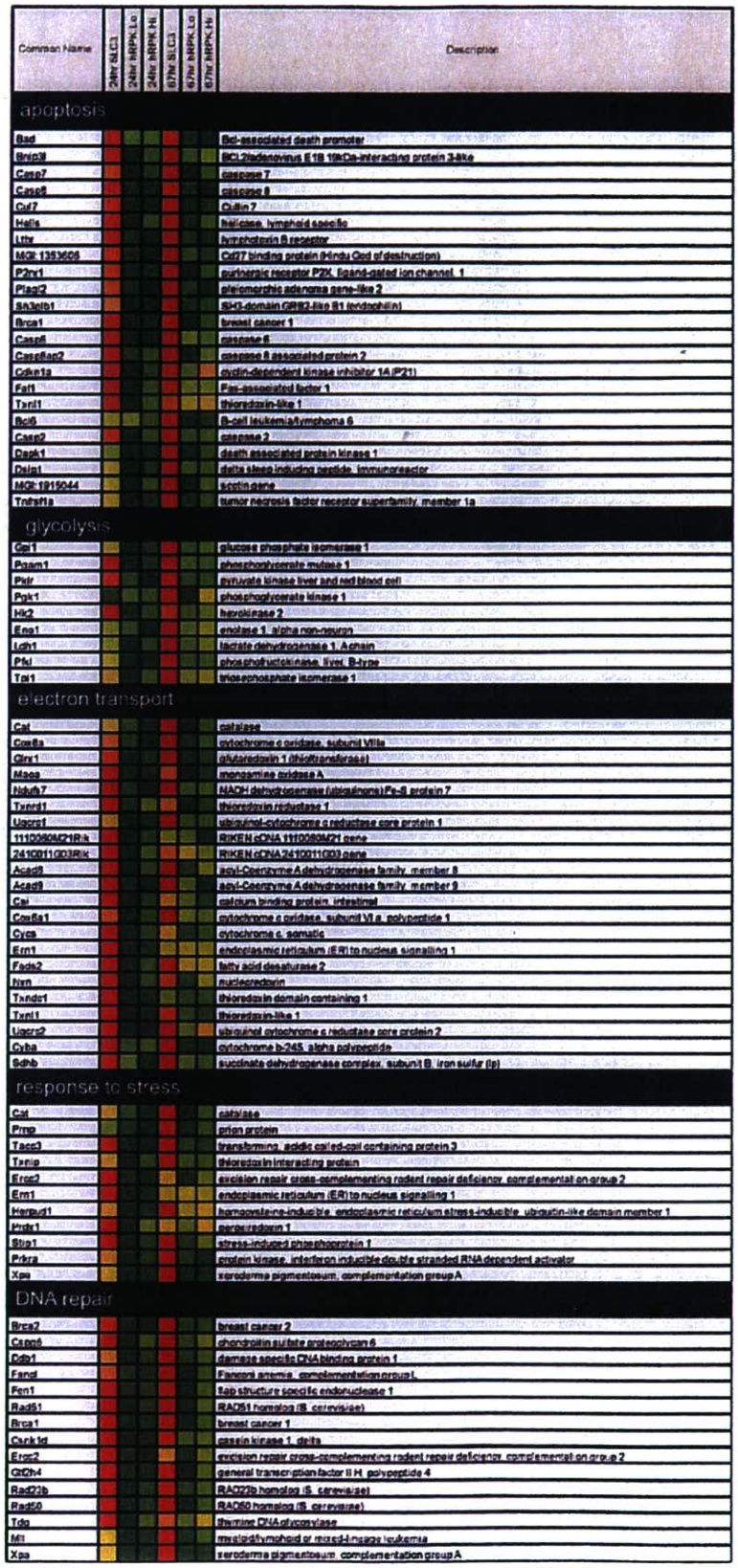


Figure 3. Genome-wide expression analysis of the glycolysis defect. Analysis was performed using the Affymetrix GeneChip Mouse Expression Array 430A, which contains about 20,000 genes. (**A**) Scatter plot between SLC3 and hRPK transfectants at 24 or 67 hours. The open circle shows the expression level of every probe set. The color shows these probabilities provided by the Affymetrix GeneChip Operation System: red means good and green means poor. The colored squares show *Bad* (red), *Bnip3* and *Bnip3l* (blue), *hif1a* (green), *Brca1* and *Brca2* (aqua), *Prdx1* (pink) and *Txnl1* (yellow), respectively. The black lines show twofold, onefold, and 0.5-fold, respectively, and the blue lines show the empirical threshold level. (**B**) The categorized aggregate graph. All probe sets were categorized by the Biological Process Ontology keywords provided by the Gene Ontology project (http://www.geneontology.org/). Up- or downregulation was determined by the spot location in the scatter plotting. Compared with the empirical threshold lines, the upper spots show upregulated genes and the lower spots show downregulated genes.

A **Down-regulated Genes**



Common Name	24hr SLC3	24hr hMPK Lo	24hr hMPK Hi	67hr SLC3	67hr hMPK Lo	67hr hMPK Hi	Description
apoptosis							
Bad							Bcl-associated death promoter
Bnip3l							BCL2/adenovirus E1B 19kDa-interacting protein 3-like
Casp7							caspase 7
Casp8							caspase 8
Cul7							Cullin 7
Hells							helicase, lymphoid specific
Lttr							lymphotoxin B receptor
MGI:1353606							Cul7 binding protein (Kindu Qod sf destruction)
P2rx1							purinergic receptor P2X, ligand-gated ion channel, 1
Ptagl2							pleiomorphic adenoma gene-like 2
Sh3glb1							(SH3-domain GRB2-like B1 (endophilin)
Brca1							breast cancer 1
Casp6							caspase 6
Casp8ap2							caspase 8 associated protein 2
Cdkn1a							cyclin-dependent kinase inhibitor 1A (P21)
Fat1							Fas-associated factor 1
Txnl1							thioredoxin-like 1
Bcl6							B-cell leukemia/lymphoma 6
Casp2							caspase 2
Dapk1							death associated protein kinase 1
Dapl1							delta sleep inducing peptide, immunoreactor
MGI:1915044							scotin gene
Tnfrsf1a							tumor necrosis factor receptor superfamily, member 1a
glycolysis							
Gpi1							glucose phosphate isomerase 1
Pgam1							phosphoglycerate mutase 1
Pklr							pyruvate kinase liver and red blood cell
Pgk1							phosphoglycerate kinase 1
Hk2							hexokinase 2
Eno1							enolase 1, alpha non-neuron
Ldh1							lactate dehydrogenase 1, A chain
Pfkl							phosphofructokinase, liver, B-type
Tpi1							triosephosphate isomerase 1
electron transport							
Cat							catalase
Cox8a							cytochrome c oxidase, subunit VIIIa
Glrx1							glutaredoxin 1 (thioltransferase)
Maoa							monoamine oxidase A
Ndufs7							NADH dehydrogenase (ubiquinone) Fe-S protein 7
Txnrd1							thioredoxin reductase 1
Uqcrc1							ubiquinol-cytochrome c reductase core protein 1
1110080M21Rik							RIKEN cDNA 1110080M21 gene
2410011G03Rik							RIKEN cDNA 2410011G03 gene
Acad8							acyl-Coenzyme A dehydrogenase family, member 8
Acad9							acyl-Coenzyme A dehydrogenase family, member 9
Cai							calcium binding protein, intestinal
Cox6a1							cytochrome c oxidase, subunit VI a, polypeptide 1
Cycs							cytochrome c, somatic
Ern1							endoplasmic reticulum (ER) to nucleus signalling 1
Fads2							fatty acid desaturase 2
Inm							nucleoredoxin
Txndc1							thioredoxin domain containing 1
Txnl1							thioredoxin-like 1
Uqcrc2							ubiquinol cytochrome c reductase core protein 2
Cyba							cytochrome b-245, alpha polypeptide
Sdhb							succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
response to stress							
Cat							catalase
Prnp							prion protein
Tacc3							transforming, acidic coiled-coil containing protein 3
Txnip							thioredoxin interacting protein
Ercc2							excision repair cross-complementing rodent repair deficiency, complemental on group 2
Ern1							endoplasmic reticulum (ER) to nucleus signalling 1
Herpud1							homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
Hspb1							heat shock protein 1
Stip1							stress-induced phosphoprotein 1
Prkra							protein kinase, interferon inducible double stranded RNA dependent activator
Xpa							xeroderma pigmentosum, complementation group A
DNA repair							
Brca2							breast cancer 2
Cspg6							chondroitin sulfate proteoglycan 6
Ddb1							damage specific DNA binding protein 1
Fancl							Fanconi anemia, complementation group L
Fen1							flap structure specific endonuclease 1
Rad51							RAD51 homolog (S. cerevisiae)
Brca1							breast cancer 1
Cdk1d							cyclin kinase 1, delta
Ercc2							excision repair cross-complementing rodent repair deficiency, complemental on group 2
Gtf2h4							general transcription factor II H, polypeptide 4
Rad23b							RAD23b homolog (S. cerevisiae)
Rad50							RAD50 homolog (S. cerevisiae)
Tdg							thymine DNA glycosylase
Mll							myeloid/lymphoid or mixed-lineage leukemia
Xpa							xeroderma pigmentosum, complementation group A

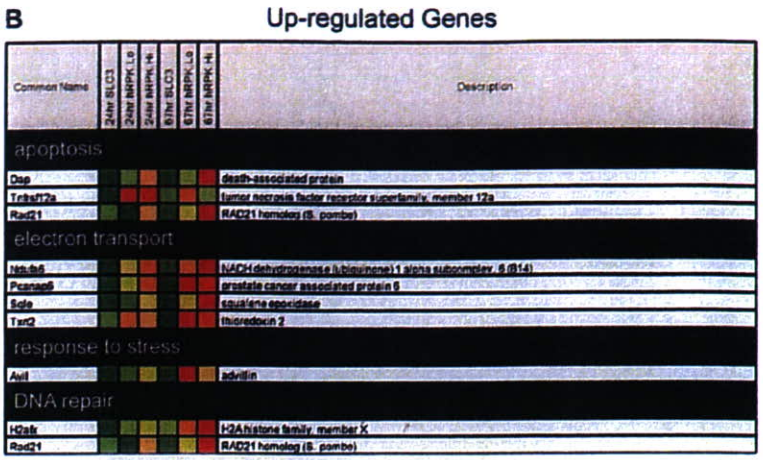


Figure 4. *Continued*

could be involved in the apoptosis induced by glycolysis defect in erythroid cells as well as in the liver.

The genes of apoptosis-inducers related to hypoxia such as *Bnip3* and *Bnip3l*, which are known as inducible genes by hypoxia-inducible factor-1α, were inactivated markedly by the forced expression of the wild-type R-PK gene. Although the extent of downregulation was smaller than for *Bnip3*, *Bnip3l* showed a significant decrease of expression by the transgene (Fig. 3A). Moreover, the downregulation was more obvious at 24 hours, suggesting that these genes may contribute to the initial response caused by a glycolytic defect. These observations strongly suggested that the apoptosis induction by the glycolysis disorder was executed by the *Bnip3-Bnip3l* signal.

It is noticeable that several genes important for responding to oxidative stress are upregulated, suggesting that R-PK deficiency might account for intracellular ROS production. This speculation is supported by the following experimental observations: Firstly, SLC3 cells were more sensitive to glycolytic inhibitions such as glucose deprivation and supplementation with 2-DG (Fig. 1), and these conditions induced ROS production detected by DCFH-DA (Fig. 5A). Apoptotic changes induced by 2-DG were partly rescued by preincubation with the glutathione precursor (Fig. 5B). Finally, transgene expression reduced intracellular ROS in an expression-level–dependent manner (Fig. 5C).

Glycolytic disorders may cause cellular conditions similar to those of hypoxia. Shim et al. [16] reported that induction of the LDH-A gene by c-Myc was advantageous to transformed cells that exist under hypoxic conditions [15]. However, glucose deprivation induces the extensive apoptosis of cells overexpressing c-Myc. Overexpression of LDH-A alone in fibroblasts is sufficient to sensitize cells to this glucose deprivation-induced apoptosis. They proposed a hypothesis that LDH-A was a downstream target of c-Myc that mediates this unique apoptotic phenotype. We noticed that pyruvate was the final product as well as the substrate of the PK and LDH reaction, respectively. Both LDH hyperactivity and PK deficiency may cause the depletion of intracellular pyruvate, suggesting that pyruvate has an important role in preventing apoptosis.

Several studies have revealed that pyruvate acts as an antioxidant and that PK has a protective role against oxidative stress in this respect. Brand et al. [17] reported that proliferating thymocytes mainly depend on energy derived from aerobic glycolysis, and that their sensitivity to 12-myristate 13-acetate–induced ROS production is much lower than that of resting thymocytes, which produce ATP mainly through oxidative phosphorylation. They suggested that pyruvate functions as an ROS scavenger, because the incubation of proliferating thymocytes with pyruvate reduced ROS formation.

The PK-overexpressing neuronal cells could attenuate oxidative stress and maintain cell viability [18]. Lee et al. [19] showed that hydrogen peroxide depleted intracellular GSH in human umbilical vein endothelial cells, and that was prevented by pyruvate but not by L-lactate or aminooxyacetate. The activation of caspases was strongly inhibited by pyruvate, but markedly enhanced by L-lactate and aminooxyacetate, implicating the redox-related antiapoptotic mechanisms of pyruvate. Myocardial ischemia-reperfusion

Figure 4. Representative list of the genes affected by the functional recovery of glycolysis. Genome-wide expression analysis was performed using Affymetrix GeneChip Mouse Expression Array 430A, which contains about 20,000 genes. In the comparison among hRPK.Hi, hRPK.Lo, and SLC3, about 6000 genes were downregulated and about 500 genes were upregulated by the functional recovery of glycolysis at 24 and/or 67 hours after regular passage. These lists contain the affected genes related to apoptosis and/or the oxidative stress response.