Bongkreikic Acid as a Warburg Effect Modulator in Long-term Estradiol-deprived MCF-7 Breast Cancer Cells

SHUSO TAKEDA^{1,2}, HIROYUKI OKAZAKI², TAICHI KUDO², KAZUHIRO KAKIZOE²,
TAICHI HIMENO², KENJI MATSUMOTO³, MITSURU SHINDO³ and HIRONORI ARAMAKI^{2,4}

¹Laboratory of Xenobiotic Metabolism and Environmental Toxicology,
Faculty of Pharmaceutical Sciences, Hiroshima International University (HIU), Hiroshima, Japan;

²Department of Molecular Biology, Daiichi University of Pharmacy, Fukuoka, Japan;

³Institute for Materials Chemistry and Engineering, Kyushu University, Kasuga, Japan;

⁴Drug Innovation Research Center, Daiichi University of Pharmacy, Fukuoka, Japan

Abstract. *Background/Aim:* An *in vitro* cell model of long-term estrogen-deprived MCF-7 (LTED) cells has been utilized to analyze the re-growth mechanisms of breast cancers treated with blockers for estrogen receptor α (ER α) signaling. Bongkreikic acid (BKA) is a natural toxin isolated from coconut tempeh contaminated with the bacterium *Burkholderia cocovenans*. *Materials and Methods:* LTED cells, MCF-7 cells and MDA-MB-231 cells were employed in the study. After treatment with BKA (chemically synthesized; purity: >98%), several biochemical analyses were carried out. *Results:* LTED cells were categorized into an oxidative phenotype. When LTED cells were treated with BKA, lactate dehydrogenase A (LDH-A)/pyruvate dehydrogenase kinase 4 (PDK4) were down-regulated, thereby prompting the aggressive use of glucose via mitochondrial oxidative phosphorylation and induction of cell death responses. These effects of BKA were not observed in the other breast cancer cells analyzed. *Conclusion:* We suggest the potential of BKA as an experimental tool for the analysis of cancer biology in LTED cells.

Approximately 70% of breast cancers express estrogen receptor α (ER α , *ESR1*) and are primarily subjected to hormone therapy. Approximately, one-third of breast cancer patients develop recurrence; however, the exact mechanism(s) responsible for the recurrence of breast cancer have not yet been established even though a number of mechanisms have

been suggested (1-3). Blockers of 17 β -estradiol (E2)/ER α signaling, such as tamoxifen, an ER α antagonist, and inhibitors of aromatase (cytochrome P450 19A1), an enzyme that catalyzes the reaction from testosterone to E2, are utilized in hormone therapy to reduce E2 levels (4). Surgical approaches, such as removal of ovaries in premenopausal women, are also effective; however, recurrence has been reported following tumor regression with reduced E2 levels. An *in vitro* cell model of long-term estrogen-deprived MCF-7 (LTED) cells has been established and utilized to investigate the regrowth mechanisms of breast cancers (1-3). Although several pathways have been suggested, including the up-regulation of insulin-like growth factor-1 (IGF-1)-mediated signaling (3), further research is required in order to obtain insight into the development of effective modalities to treat breast tumors.

Epidemiological experiments have indicated that the risk of developing breast cancer is higher in women who consume high-fat diets than in those who consume low-fat diets and fatty acid (FA) compositions have been suggested as key determinants for the outcome of breast cancer (5). ω -6 polyunsaturated FAs, such as linoleic acid (LA, C18:2) and arachidonic acid (C20:4), are known to exert strong tumor-stimulating effects, while saturated FAs, such as palmitic acid (PA, C16:0), have inhibitory effects on breast cancer cells (6, 7). The mechanisms by which these FAs modulate the proliferation of breast cancer cells remain currently unknown. However, a previous study reported that breast cancer cells overexpress FA-metabolizing enzymes, such as 15-lipoxygenase (15-LOX) and also that FAs may be metabolized by 15-LOX/Cu²⁺-mediated oxidation, resulting in the overstimulation of breast cancer cell proliferation (7).

Bongkreikic acid (BKA), a poisonous antibiotic (a natural toxin), was first isolated from coconut tempeh contaminated with the bacterium *Pseudomonas cocovenans*. The definition of a FA is a carboxylic acid (-COOH) with a long hydrocarbon

Correspondence to: Hironori Aramaki, Ph.D., Department of Molecular Biology, Drug Innovation Research Center, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan. Tel: +81 925410161, Fax: +81 925535698, e-mail: haramaki@daiichi-cps.ac.jp

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chain; when the structure of BKA was examined, the molecule was revealed to be a branched polyunsaturated long chain with a tricarboxylic acid (Figure 1) and resembled the structures of polyunsaturated FAs, such as LA and AA. BKA was initially established as an inhibitory molecule for mitochondrial adenine nucleotide translocase (ANT), also known as the ADP/ATP translocator, a component of the mitochondrial permeability transition pore (MPTP), and used as an abrogator of apoptotic cell death (8-10) in some settings. We recently demonstrated that BKA is a highly selective stimulator of peroxisome proliferator-activated receptor γ (PPAR γ) activity (11); however, other potential biological activities of BKA remain currently unknown.

The human breast cancer cell lines, ER α -negative MDA-MB-231 and -positive MCF-7 have been used to study the Warburg effect because they have glycolytic (*i.e.*, aerobic glycolysis) and oxidative (*i.e.*, mitochondrial oxidative phosphorylation; OXPHOS) phenotypes, respectively, for the production of energy for cellular processes (12, 13). Since BKA was shown to be “resistant” to 15-LOX and Cu²⁺-mediated oxidation, in contrast to LA and AA (11, 14, 15), and because rosiglitazone, an agonist of PPAR γ , attenuates IGF-1 receptor-mediated survival signaling *in vitro* and *in vivo* (16, 17), BKA has potential as an agent for the treatment of LTED cells. BKA may be obtained from commercial sources; however, its purity is generally low (approximately 90-95%). We originally established synthetic methods for BKA with high purity (>98%) (18). In the present study, we investigated whether BKA negatively affects the viability of LTED cells by comparing its effects on other breast cancer cell lines, namely, MCF-7 cells (*i.e.*, the parental cells of LTED cells) and MDA-MB-231 cells. The results obtained showed that (i) BKA specifically induced cell death responses in LTED cells and (ii) BKA selectively decreased pyruvate dehydrogenase kinase 4 (PDK4) and lactate dehydrogenase A (LDH-A) levels in LTED cells, thereby prompting these cells to use glucose as an energy source through OXPHOS. Collectively, these results indicate that BKA has potential as a modifier of the Warburg effect in LTED cells.

Materials and Methods

Reagents. BKA and BKA derivatives (BKA-2 and BKA-3) were synthesized according to established methods (18, 19). BKA-1' and BKA-4 were newly synthesized according to previously described methods (18, 19). The purities of these compounds were found to be >98% by high-performance liquid chromatography (HPLC) and column chromatography. AA, LA and PA were purchased from Cayman Chemical (Ann Arbor, MI, USA) (purity: >98%). 2-Hydroxy PA (2-OH PA) was obtained from Tokyo Chemical Industry (Tokyo, Japan) (purity: >97%). All other reagents were of analytical grade, commercially available and used without further purification.

Cell cultures. Cell culture conditions and methods were performed as described previously (20, 21). Briefly, the human breast cancer cell lines, MDA-MB-231 and MCF-7 (obtained from the American Type Culture Collection, Rockville, MD, USA) were routinely grown in phenol red-containing minimum essential medium α (MEM α) (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5% fetal bovine serum, 100 U/ml of penicillin and 100 μ g/ml of streptomycin in a humidified incubator with an atmosphere of 5% CO₂ at 37°C. Prior to the chemical treatments, medium was changed to phenol red-free MEM α supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/ml of penicillin and 100 μ g/ml of streptomycin (DCC-medium). In order to establish LTED cells using parent MCF-7 cells, LTED cells were derived from MCF-7 cells grown for 6 months in E2-depleted medium (*i.e.*, DCC-medium) based on a previous study (1). Cultures of approximately subconfluence (close to 60% confluence) in a 100-mm Petri dish were used to seed for experiments on MTS, cell viability, mRNA expression and a transfection analysis (dual-luciferase assay).

Formazan formation analysis (MTS assay). In the MTS assay, cells were seeded on 96-well plates at a density of 5×10^3 cells/well and FAs and BKA (individual concentrations were indicated in the Figures) were introduced 4 h after plating. After a 48-h incubation, cell viability was analyzed using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA), according to the manufacturer's instructions. Test chemicals were prepared in appropriate organic solvents, including dimethyl sulfoxide (DMSO) or ethanol. Control incubations contained equivalent additions of solvents with no measurable influence of vehicle on the formation of formazan at the final concentrations used.

Live/dead cell analysis. MCF-7 and LTED cells were seeded on 96-well plates at densities of 5×10^3 cells in 200 μ l cell culture medium and BKA (0.01, 1 and 25 μ M) was introduced 4 h after seeding. After a 48-h incubation, live and necrotic cells were analyzed using Live/Dead Cell Staining Kit II (PromoKine, Heidelberg, Germany), according to the manufacturer's instructions. The fluorescence of calcein-AM and ethidium homodimer III (EthD-III) was measured using the GloMax-Multi Detection System (Promega). In the morphological examination of LTED cells, images were obtained using a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany) and captured with a Pixera[®] Penguin 600CL Cooled CCD digital camera (Pixera Co., Los Gatos, CA, USA). Data were processed using Pixera Viewfinder 3.0 software (Pixera Co.). Breast cancer cells were plated on 6-well plates. Three areas with approximately equal cell densities were identified in each well and images of each of these areas were captured.

Analysis of LDH-A levels by a reverse transcription polymerase chain reaction (RT-PCR). Total RNA was prepared from MDA-MB-231, MCF-7 and LTED cells using the RNeasy kit (Qiagen, Inc., Hilden, Germany) and purified with RNeasy/QIAamp columns (Qiagen, Inc.) and cDNA (cDNA) synthesis. RT and PCR were subsequently performed using the SuperScript One-Step RT-PCR System with Platinum Taq polymerase (Invitrogen). The primers used were as follows: *LDH-A* (sense), 5'-GCA GCC TTT TCC TTA GAA CAC-3'; *LDH-A* (antisense), 5'-AAT CCA GAT TGC AAC CGC TTC-3'. Primers for the PCR of β -actin (*ACTB*) were taken from a previous study (21). PCR was performed under conditions producing template

Bongkrekkic acid (BKA)

Figure 1. Chemical structure of BKA. BKA has a branched polyunsaturated long chain with a tricarboxylic acid and resembles the structures of polyunsaturated fatty acids.

quantity-dependent amplification over 40 cycles. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate EDTA buffer and stained with ethidium bromide. When the RT reaction was omitted, no signal was detected in any of the samples. *ACTB* was used as an internal control for RT-PCR. The quantification of band intensities was performed using ImageJ 1.46r software (<http://imagej.nih.gov/ij/>).

Real-time RT-PCR analysis. Total RNA was prepared from MDA-MB-231, MCF-7 and LTED cells using the RNeasy kit (Qiagen, Inc.) and purified using RNeasy/QIAamp columns (Qiagen, Inc.). In the real-time RT-PCR analysis of *LDH-A*, *PDK4*, *Topo II α* , *Ki-67*, *CPT1A* and *ACTB*, cDNA was prepared via the RT of total RNA using the ReverTra Ace[®] qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Real-time quantitative RT-PCR assays were performed with FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) and LightCycler[®] Nano (Roche Diagnostics, Mannheim, Germany). The primers used were as follows; *LDH-A* (sense), 5'-GCA GCC TTT TCC TTA GAA CAC-3'; *LDH-A* (antisense), 5'-AAT CCA GAT TGC AAC CGC TTC-3'; *PDK4* (sense), 5'-AGA GCC TGA TGG ATT TGG TG-3'; *PDK4* (antisense), 5'-GCT TGG GTT TCC TGT CTG TG-3'; *Topo II α* (sense), 5'-CTA TTG AAG AAC TGG CTC CAA-3'; *Topo II α* (antisense), 5'-CTT TAA ACA GCC TAC GTG GTC-3'; *Ki-67* (sense), 5'-TAT CCA GCT TCC TGT TGT GTC-3'; *CPT1A* (sense), 5'-TC CAG TTG GCT TAT CGT GGT G-3'; *CPT1A* (antisense), 5'-CTA ACG AGG GGT CGA TCT TGG-3'. The primers for the PCR of *ACTB* were taken from a previous study (22). The reaction conditions for all mRNAs were 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 58°C for 10 s and 72°C for 15 s. *LDH-A*, *PDK4*, *Topo II α* , *Ki-67* and *CPT1A* mRNA levels were normalized to the corresponding *ACTB* mRNA levels.

Transfection and luciferase reporter assay (dual-luciferase assay). The day before transfection, MCF-7 cells were seeded (5×10^4 cells/well) on 24-well plates containing MEM α medium. The transfection of each expression plasmid was performed using Lipofectamine[®] LTX with PLUS[™] reagent (Invitrogen) according

Figure 2. Transportation of cytosolic NADH into mitochondria and principle of the MTS assay. NADH, a reducing equivalent, is produced in the cytosol, followed by transportation into mitochondria. After that, MTS reagent (Owen's reagent) may be reduced by NADH, which results in the formation of a colored formazan product in mitochondria; MTS may be selectively cleaved by mitochondrial succinate dehydrogenase.

to the manufacturer's instructions. The maximal transcriptional efficiencies of the human PPAR γ expression plasmid in combination with the human retinoid X receptor α (RXR α) plasmid were 100 ng and 100 ng, respectively, in the transfections. DNA mixtures of 300 ng of the PPRE-Luc plasmid containing the rat acyl-CoA oxidase PPRE were co-transfected with 20 ng of the Renilla luciferase reporter plasmid (pRL-CMV) in 24-well plates. All plasmid concentrations were equalized with the pcDNA3.1 vector. The expression plasmids of human PPAR γ , RXR α and the PPRE reporter construct were gifts from Dr. Curtis J. Omiecinski (Pennsylvania State University, PA, USA) (11, 23). At 24 h post-transfection, cells were washed with phosphate-buffered saline and changed to MEM α without phenol red supplemented with 5% serum, followed by a treatment with BKA and its derivatives (BKA-1', BKA-2, BKA-3 and BKA-4) for 24 h. After being treated with the compounds, cell extracts were prepared with 100 μ l of passive lysis buffer (Promega) and 20 μ l of the extracts was used for the firefly luciferase and Renilla luciferase assays (Dual-Luciferase Reporter Assay System) by the GloMax-Multi Detection System (Promega). The ratio of firefly luciferase activity (expressed from reporter plasmids) to Renilla luciferase activity (expressed from pRL-CMV) in each sample served as a measure of normalized luciferase activity.

Assay for the formation of conjugated dienes. Experiments were performed based on previously described procedures (11, 15). BKA or LA in the presence of copper (Cu²⁺, CuSO₄) was incubated for various times in spectrophotometric cuvettes (1.0-cm light path) in 100 mM borate buffer at pH 9.0 at room temperature. Absorbance at 234 nm was periodically recorded after the addition of copper. Results were expressed as an increase in absorbance from the reaction mixture at time zero.

Data analysis. Half-maximal effective concentration (EC₅₀) values were obtained using SigmaPlot 11[®] software (Systat Software, Inc., San Jose, CA, USA). Differences were considered significant when the *p*-value was calculated as <0.05. Significant differences between two independent groups were calculated by the Student's *t*-test. Other statistical analyses were performed by Scheffé's F test, a *post-hoc* test for analyzing the results of ANOVA. These calculations were performed using Statview 5.0J software (SAS Institute Inc., Cary, NC, USA).

Figure 3. Effects of BKA on formazan formation in three breast cancer cell lines: MDA-MB-231, MCF-7 and LTED cells. (A and B, right panel) Cells were exposed to BKA for 48 h at concentrations ranging between 0.01 and 50 μ M to determine EC_{50} values in formazan formation. After exposures, formazan formation was measured by MTS assay according to the methods described in the Materials and Methods. (B, left panel) Comparison of basal formazan formation between MCF-7 and LTED cells. Cells were used for the MTS assay after seeding for 48 h. Data are expressed as a percentage of the vehicle-treated group (indicated as Ctl.) or MCF-7 cells, as the mean $\pm$ SE (n=6). *Significantly different ($p<0.05$) from the vehicle-treated control or MCF-7 cells.

Results and Discussion

Effects of BKA on formazan formation in $ER\alpha$ -positive (MCF-7 and LTED cells) and $ER\alpha$ -negative MDA-MB-231 cells. We first performed an MTS assay using a reagent of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Owen's reagent] (24) to analyze whether BKA (Figure 1) and FAs affect the formation of

formazan in breast cancer cells. The MTS assay utilizes reducing equivalents, such as the co-enzyme nicotinamide adenine dinucleotide phosphate reduced form (NADH) to convert MTS into a colored formazan product; MTS may be selectively cleaved by mitochondrial succinate dehydrogenase, which is a component of complex II (Figure 2). The MTS assay is generally used as an alternative method for [3 H]thymidine incorporation. As shown in Figure 3, we investigated the effects

Figure 4. BKA-mediated decreases in living cell numbers: a comparison of LTED cells and parent MCF-7 cells. (A, B and D) MCF-7 and LTED cells were exposed to BKA (0.01, 1 and 25 μM) for 48 h. After the treatments, live and necrotic cells were analyzed using calcein-AM and EthD-III, respectively. The fluorescence of calcein-AM/EthD-III was measured by the GloMax-Multi Detection System. (C) LTED cells were treated with vehicle (Ctl.) and 25 μM BKA for 48 h prior to the morphological examination. Representative data images are shown. Images were taken at $\times 200$ magnification. Data are expressed as a percentage of the vehicle-treated group (indicated as Ctl.), as the mean $\pm$ SE ($n=6$). *Significantly different ($p<0.05$) from the vehicle-treated control.

of BKA on the formation of formazan in three breast cancer cell lines: MDA-MB-231, MCF-7 and LTED. Although BKA positively enhanced the formation of formazan in MCF-7 cells ($EC_{50}=34.14$ μM), the molecule weakly stimulated its formation in MDA-MB-231 cells with an EC_{50} value of >50 μM (Figure 3A). When MCF-7 cells were compared with LTED cells, the basal formation of formazan was approximately 1.5-fold higher in LTED cells than in parent MCF-7 cells (Figure 3B, left panel), even though these cells were seeded at the same cell number (5×10^3 cells/well). In

the trial with LTED cells, BKA effectively stimulated formazan formation with an EC_{50} value of 2.58 μM (Figure 3B, right panel). Thus, BKA appears to preferentially accelerate the formation of formazan in breast cancer cells, particularly ER α -positive LTED cells.

BKA-mediated decreases in living cells: a comparison of LTED cells and parent MCF-7 cells. The enhanced formation of formazan is generally considered to reflect the number of living cells (*i.e.*, cell viability). If this is the case

in LTED cells treated with BKA, results using other approaches to examine living cells may be the same or similar to those obtained by the MTS assay. We utilized a calcein (calcein AM) probe and trypan blue dye to detect live cells. This calcein AM probe readily passes through the membranes of “viable cells”. After being transported into live cells, calcein is hydrolyzed by cytosolic esterases into a green-fluorescent calcein. Since dead cells lack esterases, live cells are selectively marked with the probe. Although BKA had no observable effects on the viability of MDA-MB-231 cells, even at 25 μ M (data not shown), it slightly reduced the number of live MCF-7 cells at concentrations up to 25 μ M (Figure 4A) and significantly reduced the number of live LTED cells in a concentration-dependent manner (Figure 4B). The BKA-mediated suppression of cell growth was detected with the trypan blue exclusion test (data not shown). In support, the morphology of LTED cells was affected (implicating cell death responses) more by BKA than vehicle-treated control cells (Figure 4C). Furthermore, an analysis of the cell death type using an EthD-III probe indicated that BKA-mediated death was not dependent on necroptotic cell death (Figure 4D).

Expression status of LDH-A in breast cancer cells and effects of BKA on the expression of LDH-A. LDH-A is one of the major isoforms of LDH expressed in breast tissues that regulates the conversion of pyruvate to lactate in the cellular glycolytic process (25). MDA-MB-231 and MCF-7 breast cancer cell lines have been used to study the Warburg effect (12, 13); highly aggressive MDA-MB-231 cells are known to obtain ATP by the glycolytic pathway, even in the presence of O_2 (*i.e.*, the “glycolytic” phenotype), which converts glucose to lactate and reduces OXPHOS activity (Figure 5A). In contrast to MDA-MB-231 cells, non-invasive/less tumorigenic MCF-7 cells utilized the OXPHOS pathway to produce ATP with lower glycolytic activity (*i.e.*, the “oxidative” phenotype) (Figure 5B). In support of the above-described phenomena, decreases in LDH-A levels correlated with increases in cytosolic NADH/NAD⁺, which is consistent with a higher mitochondrial respiration rate (26) (Figures 5A and 4B). Thus, we focused on the expression status of LDH-A in the three breast cancer cells. Semiquantitative and real-time RT-PCR analyses indicated that *LDH-A* expression was detected in the following order: MDA-MB-231 cells (5.79-, 2.3-fold) >> MCF-7 cells (1.0-fold)=LTED cells (0.76-, 1.3-fold) (Figure 6A). Therefore, LTED cells were categorized into the “oxidative phenotype”, similar to the parent MCF-7 cells. We then investigated the effects of BKA and FAs (PA and AA) on the expression of LDH-A in breast cancer cells; BKA and FA concentrations were fixed at 25 μ M based on the results shown in Figure 6. AA did not exert any modulating activity on any of the breast cancer cells examined. In spite of the cell type, PA up-

Figure 5. Schematic representation of differences between aerobic glycolysis and oxidative phosphorylation (OXPHOS). (A) Cancer cells, including MDA-MB-231 cells, rely on aerobic glycolysis to generate ATP. MDA-MB-231 cells expressed higher levels of LDH-A and PDK4 than MCF-7 and LTED cells. (B) In contrast to MDA-MB-231 cells, MCF-7 and LTED cells rely primarily on mitochondrial OXPHOS to generate ATP.

regulated the expression of *LDH-A*, whereas BKA significantly down-regulated its expression in LTED cells (0.66-fold) compared to control (1.0-fold) (Figures 6B–D).

Expression status of PDK4 in breast cancer cells and effects of BKA on the expression of PDK4. Previous studies reported that cellular glucose oxidation is decreased by FAs due to the induction of PDK4, a key enzyme for the regulation of glucose (carbohydrate) oxidation *via* the inhibition of the pyruvate dehydrogenase complex (PDH) in mitochondria (27, 28) (see Figure 5A). Taken together with the results described in Figure 6, BKA appears to modulate the expression status of “key enzymes” responsible for the metabolic fate of glucose. The results of the real-time RT-PCR analysis demonstrated that the basal expression of *PDK4* varied markedly among the three breast cancer cells tested; MDA-MB-231 cells (2.18-fold) expressed the highest levels of *PDK4*, followed by MCF-7 cells (1.0-fold) and LTED cells (0.08-fold) (Figure 7A). Thus, LTED cell growth appears to be highly dependent on

Figure 6. Expression status of LDH-A in breast cancer cells and effects of BKA on the expression of LDH-A. (A) Semi-quantitative RT-PCR (A, left panel) and real-time RT-PCR (A, right panel) analyses of LDH-A basally expressed in individual MDA-MB-231, MCF-7 and LTED cells. Beta-actin (ACTB) was used as an internal loading control. Band intensities were quantified by densitometry (ImageJ 1.46r software) and normalized to ACTB levels (A, left panel). (B-D) Real-time RT-PCR analyses of LDH-A in MDA-MB-231, MCF-7 and LTED cells 48 h after the treatment with vehicle (indicated as Ctl.), PA, LA or BKA (25 μ M). Data are expressed as a fold change or induction from MCF-7 cells or the vehicle-treated control, as the mean $\pm$ S.E. (n=3). *Significantly different ($p < 0.05$) from each cell or the vehicle-treated control. N.S., Not significant.

glucose (*i.e.*, glucose oxidation) as an energy source. We then investigated the effects of BKA and two FAs (PA and AA) on the three breast cancer cells. As expected, the expression of *PDK4* was significantly stimulated by the two FAs in MDA-

MB-231 cells. However, AA up-regulated the expression of *PDK4*, whereas PA down-regulated it in MCF-7/LTED cells (Figures 7B-D). It is important to note that, in contrast to the effects of FAs, BKA “selectively” down-regulated the

Figure 7. Expression status of *PDK4* in breast cancer cells and effects of BKA on the expression of *PDK4*. (A) Real-time RT-PCR analyses of *PDK4* basally expressed in individual MDA-MB-231, MCF-7 and LTED cells. (B-D) Real-time RT-PCR analyses of *PDK4* in MDA-MB-231, MCF-7 and LTED cells 48 h after the treatment with vehicle (indicated as Ctl.), PA, LA or BKA (25 μ M). Data are expressed as a fold change or induction from MCF-7 cells or the vehicle-treated control, as the mean $\pm$ S.E. (n=3). *Significantly different ($p < 0.05$) from each cell or the vehicle-treated control.

expression of *PDK4* in LTED cells (Figure 7D) and, as shown in Figure 6D, *LDH-A* in LTED cells was identified as a target of BKA. Thus, BKA-mediated decreases in *PDK4*/*LDH-A* levels may relieve the inhibition of PDH, thereby prompting the aggressive use of glucose.

Expression status of *Topo II α* in breast cancer cells and effects of BKA on the expression of *Topo II α* . We measured the expression pattern of the proliferation marker *Topo II α* in the three breast cancer cells. Real-time RT-PCR analyses revealed that the strongest expression of *Topo II α* was detected in MDA-MB-231 cells (1.3-fold), followed by MCF-7 cells (1.0-fold) > LTED cells (0.54-fold). Although the reason why *Topo II α* was more weakly expressed in LTED cells than in parent MCF-7 cells remains currently unclear, it is speculated that highly aggressive MDA-MB-231 breast cancer cells express the highest levels of *Topo II α* (Figure 8A). This expression order of *Topo II α* was similar to that of *PDK4* (Figure 7A). Consistent with the results shown in Figure 8B, PA at 25 μ M

decreased the viability of MDA-MB-231 cells (data not shown) (6). When the effects of BKA were investigated in more detail, we found that this molecule down-regulated the expression of *Topo II α* in LTED cells only; this modulative effect by BKA was only observed for the expression of *PDK4* in LTED cells (Figures 8B-D; see also Figures 6 and 7). In order to further support the modulation of *Topo II α* by BKA in LTED cells, we focused on another proliferation marker of cancer cells, Ki-67 (29). Real-time RT-PCR results for the expression of Ki-67 were similar to those of *Topo II α* (0.41 ± 0.014 vs. Ctl.=1.0, $p < 0.05$), indicating BKA-selective inhibitory effects on the proliferation of LTED cells. Inversely related to the formation of formazan by BKA (Figure 2B), proliferation markers' (*Topo II α* and Ki-67) levels and living cell numbers were reduced by BKA in LTED cells (Figures 3B and 8D).

Effects of simplified BKA analogs on formazan formation and *PPAR γ* -mediated transcriptional activity. As we reported previously, BKA has been established as a selective activator

Figure 8. Expression status of *Topo IIα* in breast cancer cells and effects of BKA on the expression of *Topo IIα*. (A) Real-time RT-PCR analyses of *Topo IIα* basally expressed in individual MDA-MB-231, MCF-7 and LTED cells. (B-D) Real-time RT-PCR analyses of *Topo IIα* in MDA-MB-231, MCF-7 and LTED cells 48 h after the treatment with vehicle (indicated as Ctl.), PA, LA or BKA (25 μ M). Data are expressed as a fold change or induction from MCF-7 cells or the vehicle-treated control, as the mean $\pm$ S.E. (n=3). *Significantly different ($p<0.05$) from each cell or the vehicle-treated control.

for PPAR γ , coupled with the up-regulation of its regulated gene, adiponectin (*ADIPOQ*), but not with fatty acid 2-hydroxylase (*FA2H*), a PPAR α -regulated gene (11, 23). After switching parent MCF-7 cells into LTED cells cultured under reduced E2 conditions, growth signaling mediated by IGF-1 (*i.e.*, mitogenic and anti-apoptotic effects) was observed (30) and, in some settings, the activation of PPAR γ by rosiglitazone suppressed the IGF regulatory system *in vitro* and *in vivo* (16). Furthermore, a strong positive association has been reported between IGF-1 levels and breast cancer (30). Based on these findings, we hypothesized that the selective anti-proliferative effects of BKA on LTED cells are based on its association with PPAR γ . As shown in Figure 9A, we originally synthesized two simplified BKA analogs, BKA-1' and BKA-4, and applied them in addition to BKA, BKA-2 and BKA-3 (11) to the formazan formation assay at 10 and 50 μ M. Of these, only BKA-3 exhibited significant, but weaker stimulating activity at 50 μ M than that of parent BKA (Figures 3B and 9B). Transcriptional experiments on PPAR γ

revealed that BKA-3 (4.86-fold) and BKA-4 (4.18-fold) in addition to BKA (2.07-fold) activated PPAR γ (Figure 9C). Although the PPAR γ activation potentials of BKA-3/BKA-4 were approximately 2-fold stronger than that of BKA, they exhibited very weak or negative effects on formazan formation. Thus, the stimulatory effects of BKA on PPAR γ are not assertively involved in LTED-directed cell death.

The IGF-1-mediated down-regulation of *PK4* has been reported (31). Thiazolidinediones (rosiglitazone and pioglitazone), which are PPAR γ agonists, have been shown to stimulate IGF-1 signaling *via* a PPAR γ -independent pathway in human aortic smooth muscle cells (32). Although this cell system is different from that used in the present study, if this signaling plays a role in the effects of BKA, BKA may utilize similar pathway(s) to thiazolidinediones in LTED cells. The capacity to transport cytosolic NADH into mitochondria is reduced in rapidly proliferating cells (33). If this finding is applied to aggressive MDA-MB-231 cells, the transporting systems for NADH in MCF-7 and LTED cells are up-regulated

Figure 9. Effects of BKA analogs on formazan formation and PPAR γ -mediated transactivation activity. (A) Chemical structures of BKA analogs (BKA-1', 2, 3 and 4). (B) LTED cells were exposed for 48 h to BKA (10 and 50 μ M). After the treatments, formazan formation was measured by the MTS assay according to the methods described in the Materials and Methods. Data are expressed as a percentage of the vehicle-treated group (indicated as Ctl.), as the mean $\pm$ SE (n=6). *Significantly different (p<0.05) from the vehicle-treated control. (C) MCF-7 cells were transiently transfected with a PPRE-luciferase reporter plasmid in combination with a pcDNA3.1 plasmid and expression plasmid of human PPAR γ . After transfection, cells were treated with vehicle (Ctl.) or BKA and its analogs (BKA-1', 2, 3 and 4) at 25 μ M. After 24 h, cells were harvested and assayed for luciferase activity and all transfections were normalized for efficiency using the internal Renilla control plasmid. Data are expressed as fold activation from the vehicle-treated control (Ctl.), as the mean $\pm$ S.E. (n=3). The results for BKA, BKA-2 and BKA-3 presented in the Figure were taken from reference 11.

(Figure 2). Taken together with findings demonstrating that extracellularly added NADH (which enters cells and reaches the OXPHOS apparatus) suppresses the proliferation of MCF-7 cells, coupled with cell death accompanying DNA fragmentation (34-36) and formazan formation (NADH production) as a hallmark of mitochondrial activity, the cell death responses induced by BKA appear to be the result of the "overactivation" of mitochondria (Figures 2, 3B, 4B, 5B and 8).

BKA is insensitive to Cu²⁺-mediated oxidation. Fatty acids are oxidized by 15-LOX and/or Cu²⁺-mediated reaction in the body; for example, LA (C18:2) may be metabolized into

($\pm$)13-HpODE (Figure 10A), a positive stimulator for breast cancer cell growth (7). When the structure of BKA was examined, it was found to have a dienylnmethylene (-CH=CH-CH=CH-CH₂-), a possible oxidizable moiety (Figure 1). Thus, we speculated that BKA may be oxidized by Cu²⁺; however, the results shown in Figure 10B revealed that BKA is insensitive to Cu²⁺, whereas LA was oxidized. The resistance of BKA to 15-LOX was also demonstrated (data not shown) (11). Furthermore, we did not detect any absorption peaks at wavelengths ranging between 200 nm and 800 nm (data not shown). Collectively, these results suggest that BKA is more stable than LA against Cu²⁺-mediated oxidation.

Figure 10. BKA was not sensitive to Cu²⁺-mediated oxidation. (A) Chemical structure of LA. (B) BKA or LA was incubated with Cu²⁺ for up to 240 s as described in the Materials and Methods. Absorbance at 234 nm was periodically recorded. Results are expressed as an increase in absorbance from the reaction mixture at time zero. The modified moiety (active methylene) in LA is indicated with an inclusion. (±)13-HpODE, an oxidized metabolite, is also shown.

Conclusion

In the present study, we did not obtain direct evidence for the interaction between the BKA-induced down-regulation of *LDH-A/PDK4* (*Topo IIα/Ki-67*) and LTED cell death; however, this is the first study to identify BKA as a highly selective modulator for the metabolic pathway in LTED cells (Figure 5B). BKA has potential as a therapeutic modality for the recurrence of breast cancers that have already been treated with blockers of 17β-estradiol/ERα signaling; however, further investigations are needed on the mechanism(s) responsible for BKA-mediated cell death in LTED cells.

Conflicts of Interest

No potential conflicts of interest were disclosed.

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