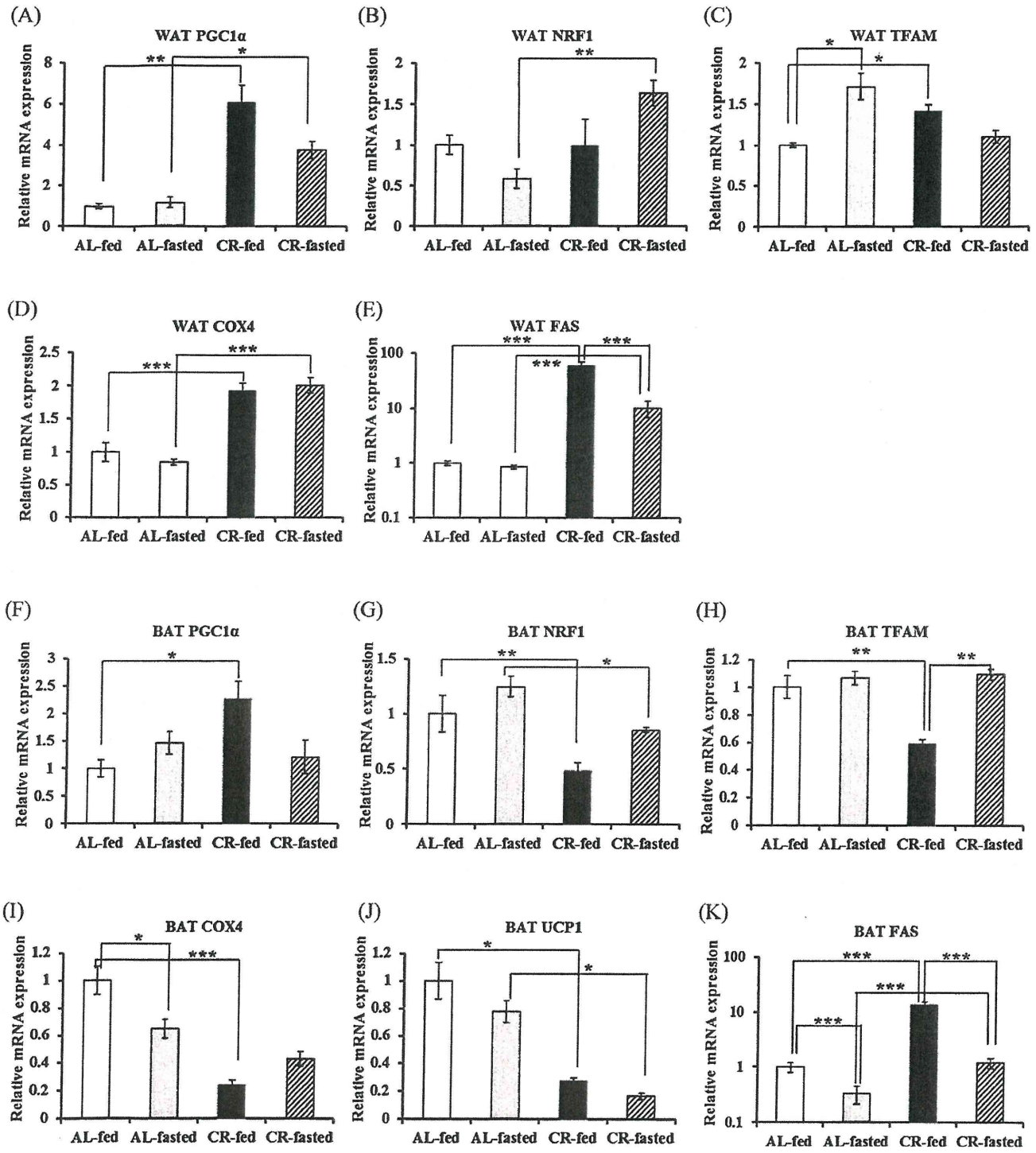


**Fig. 5.** Effects of CR on the expression of genes involved in mitochondrial biogenesis and fatty acid biosynthesis in WAT (A–E) and BAT (F–K). The mRNA levels of PGC1α (A and F), NRF1 (B and G), TFAM (C and H), COX4 (D and I), UCP1 (J) and FAS (E and K) were determined by real-time RT-PCR. The average intensity of each product was relative to the control gene TBP. Values shown in all panels are means ± SEM of three to five animals in each group, and are expressed as the fold change relative to the mean value of AL-fed rats. $^{*}p < 0.05$, $^{**}p < 0.01$. $^{***}p < 0.001$ by Tukey's *t*-test.

Fatty acid synthase (FAS) plays a central role in *de novo* lipogenesis (Griffin and Sul, 2004). In WAT and BAT, the mRNA levels of FAS were up-regulated by CR in both fed and fast states (Fig. 5E and K). Therefore, it is likely that CR activates fatty acid biosynthesis in both WAT and BAT. In AL rats, however, fasting suppressed FAS expression in BAT but not in WAT.

## 4. Discussion

### 4.1. WAT response to CR

In WAT of CR rats, the adipocytes had a relatively small and uniform size. The morphological change induced by CR was

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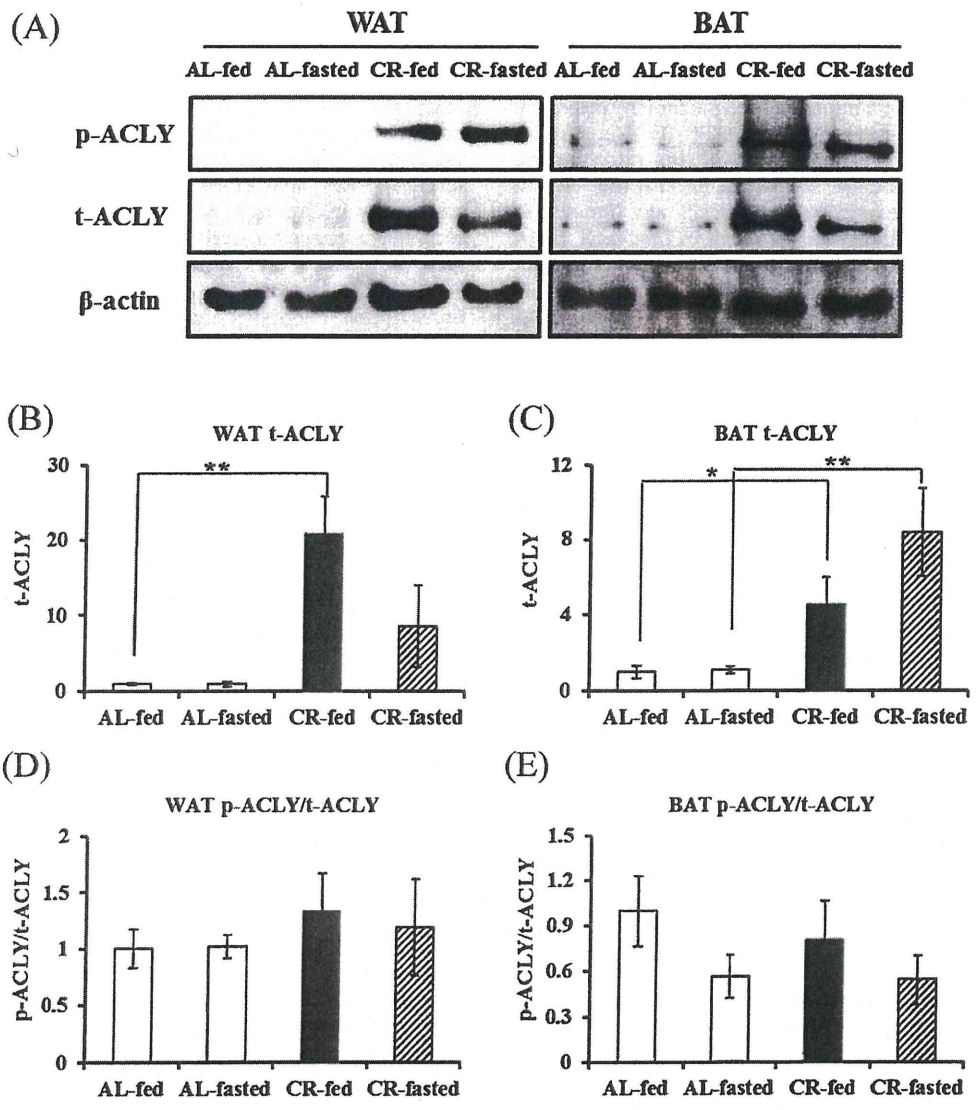


**Fig. 6.** CR-associated alteration of phosphorylation levels of ACLY in WAT and BAT. Protein samples were extracted from WAT and BAT of AL-fed, AL-fasted, CR-fed and CR-fasted rats. Western blot analysis of total ACLY (t-ACLY) and phosphorylated form of ACLY (p-ACLY) was performed using the chemiluminescence method. The specific proteins were visualized using an LAS3000 image analyzer. Both β-actin and the intensity of CBB stain were used as a normalization control. Western blot analysis was performed in duplicate or triplicate from each sample with biological repeats of $n = 5$ for each group. A representative gel image of the Western blot is shown (A). Densitometry data of t-ACLY (B and C) and p-ACLY/t-ACLY (D and E) were measured, and are shown for WAT (B and D) and BAT (C and E).

associated with reduced TG content. In contrast, adipocytes of various sizes, including large hypertrophic adipocytes, were found in AL rats. These findings confirm previous data (Higami et al., 2004; Zhu et al., 2007). In general, excessive energy associated with excessive food intake is accumulated in adipocytes in the form of TG. The excessive TG accumulation is initially compensated for by increased adipocyte size. Further TG accumulation induces an increased number of adipocytes *via* proliferation of preadipocytes (Sakai et al., 2007). Therefore, it is likely that in AL rats the excessive energy supply was compensated for by both increased size and increased number of adipocytes in WAT. In CR rats, such compensation is not required due to the lower energy supply. It is well known that small adipocytes secrete more adiponectin and less pro-inflammatory cytokines, such as TNFα and leptin (Higami et al., 2005; Zhu et al., 2007). In addition, small adipocytes are generally more sensitive to insulin and act as powerful buffers absorbing lipids in the postprandial period. Hence, it appears that the small-sized white adipocytes observed in CR rats are more sensitive to insulin, secrete more adiponectin and less pro-inflammatory adipokines, and have a more powerful buffering activity for lipids (Frayn, 2002; Higami et al., 2005; Zhu et al., 2007).

All five proteins shown to be up-regulated in WAT by CR are metabolic enzymes involved in lipid metabolism (ACLY, MAOX and ACADL) and glucose metabolism (ODPB and PYC). Previously, it had been demonstrated by DNA microarray analysis that most genes up-regulated by CR are involved in metabolic processes (Higami et al., 2004, 2006b). Using proteome analysis in WAT, we showed that CR up-regulates three mitochondrial enzymes: ACADL, ODPB and PYC. ACADL is involved in β-oxidation of long-chain fatty acids (Ikeda et al., 1985). ODPB is an E1 component beta subunit of the pyruvate dehydrogenase (PDH) complex (Huh et al., 1990). The mitochondrial PDH complex catalyzes the conversion of pyruvate to acetyl-CoA and $CO_2$, linking glycolysis to the Krebs cycle and fatty acid biosynthesis (Sugden and Holness, 2003). PYC catalyzes the carboxylation of pyruvate to oxaloacetate (Jitrapakdee et al.,

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2006). Thus, our finding supports previous reports showing that CR promotes mitochondrial biogenesis in WAT (Anderson and Prolla, 2009; Nisoli et al., 2005). To confirm the CR-associated mitochondrial activation, we analyzed mitochondrial function by measuring mtDNA contents, mRNA levels of PGC1α, NRF1, TFAM and COX4, and the activities of CS and Complex IV of the mitochondrial respiratory transport chain. We found that in both the fed and fasted states, CR increased mtDNA content, CS and Complex IV activities, and mRNA expression levels of PGC1α and COX4. Moreover, CR enhanced the expression of NRF1 in the fasted state and of TFAM in the fed state. Thus, CR activates various mitochondrial metabolic processes (Anderson and Prolla, 2009; Higami et al., 2004; Nisoli et al., 2005). The activity scale of CS was higher in BAT compared with WAT (Fig. 4C and D); however, when examining the activity of Complex IV this difference was markedly larger (Fig. 4E and F, note the *Y*-axis scales). Therefore, the Krebs cycle may have a more dominant function than the respiratory transport chain in WAT compared with BAT.

Proteome analysis also revealed that the expression of both ACLY and MAOX, which are cytoplasmic enzymes involved in lipid metabolism, was increased by CR. ACLY catalyzes the conversion of citrate and CoA to acetyl-CoA and oxaloacetate (Ramakrishna and Benjamin, 1979). Therefore, it is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA. The product, acetyl-CoA, serves as a substrate for important biosynthetic pathways, including lipogenesis. MAOX catalyzes oxidative decarboxylation of malate to pyruvate (Taroni and Di Donato, 1988). Importantly, MAOX generates NADPH, which is a critical fatty acid biosynthesis substrate (Taroni and Di Donato, 1988). FAS plays a central role in *de novo* fatty acid biosynthesis by converting acetyl-CoA and malonyl-CoA into the final end product, palmitate, which can subsequently be esterified into triacylglycerols and then stored in adipose tissue (Griffin and Sul, 2004). The mRNA level of FAS was up-regulated by CR in WAT. Thus, our data suggest that CR activates fatty acid biosynthesis in both the fed and fasted states. Recent proteome analysis also found that CR enhanced the expression of MAOX and FASN in WAT of rats at 24 months of age (Valle et al., 2010). This suggests that maintaining enhanced *de novo* fatty acid biosynthesis throughout life may be an important beneficial action of CR.

Proteome analysis also found that CR suppressed the expression of HSPB1. It has been reported that HSPB1, a molecular chaperone that protects partially mis-folded proteins (particularly during oxidative stress), is up-regulated by a high-fat diet (Balwierz et al., 2009; Dosh et al., 2010). Our finding is consistent with the interpretation that the expression of this protein is reduced because CR attenuates oxidative stress and thus the requirement for this chaperone may be decreased (Wang and Paigen, 2005).

### 4.2. BAT response to CR

In contrast to WAT, CR enlarged the lipid droplets in BAT. Because white adipocytes tend to infiltrate into BAT with aging, the lipid droplets observed in BAT were probably derived from both unilocular lipid droplets of infiltrating small white adipocytes and multilocular lipid droplets in brown adipocytes. In fact, CR showed some evidence of up-regulation of the mRNA level of leptin (a white adipocyte marker) in BAT in both the fed and fasted states, although this was not statistically significant (unpublished data). Together, these results suggest that CR could promote the infiltration of white adipocytes into BAT as well as the accumulation of TG in multilocular lipid droplets in brown adipocytes.

Proteome analysis of BAT demonstrated reduced expression of four mitochondrial enzymes (COX5B, NAUV1, ODBB and SUCB1) in response to CR; two out of these proteins (COX5B, NAUV1) are involved in the electron transport chain. Consistent with the observed proteome profiles, CR significantly suppressed mRNA levels of COX4 in the fed state. Moreover, CR markedly reduced mtDNA content and mRNA levels of NRF1 and TFAM in the fed state, and mRNA levels of UCP1 in both fed and fasted states. In contrast, the level of PGC1α mRNA was up-regulated by CR in the fed state. Fasting did not change mtDNA content, or the expression levels of NRF1 and TFAM, in AL rats, but it increased these parameters in CR rats. In BAT, mtDNA content roughly correlated with the expression levels of NRF1 and TFAM, but inversely correlated with the expression of PGC1α. It is well known that PGC1α is a master regulator of mitochondrial biogenesis and UCP1 transcription (Puigserver and Spiegelman, 2003). However, CR- or fasting-induced expression of PGC1α did not correlate with the expression of UCP1. Further examination is required to elucidate this discrepancy.

In BAT, CR did not markedly affect the activities of either CS or Complex IV. Moreover, CR suppressed the expression of mitochondria-related genes including NRF1, TFAM COX4 and UCP1, suggesting that, in contrast to WAT, CR does not activate mitochondrial function. However, as was observed in WAT, levels of the cytoplasmic enzymes ACLY and MAOX (Berwick et al., 2002; Ramakrishna and Benjamin, 1979; Taroni and Di Donato, 1988), and the mRNA level of FAS were up-regulated by CR. Furthermore, we found that CR induced ACLY phosphorylation, suggesting that CR promotes fatty acid biosynthesis in BAT as well as in WAT. CS catalyzes the reaction from acetyl-CoA and oxaloacetate to citrate, which is the first step of the Krebs cycle (Alp et al., 1976; Wiegand and Remington, 1986). Citrate is used as a substrate for the Krebs cycle in mitochondria, as well as a substrate for ACLY when exported from the mitochondria to the cytoplasm. Therefore, it may be important to maintain CS activity to promote lipogenesis even under the CR condition. In contrast, fasting reduced CS activity and the mRNA level of FAS, suggesting that fasting might suppress lipogenesis by reducing the citrate supply from mitochondria.

As mentioned above, our data suggest that CR activates various mitochondrial functions, including the Krebs cycle and the electron transport chain, in WAT. It also accelerates fatty acid biosynthesis. We consider, therefore, that CR animals use glucose predominantly in glucose-dependent organs such as the central nervous system after feeding. To use energy effectively under the condition of energy shortage, residual glucose could be converted to more energy-dense fatty acids in WAT. Then, the newly generated fatty acids might be stored in the form of TG in WAT and BAT, and/or be supplied to and used in non-glucose-dependent organs. In other words, it is likely that WAT in CR rats functions as an energy transducer from glucose to more energy-dense lipids and not as an energy storage system (Fig. 7A). In BAT, CR suppresses mitochondrial function, but activates fatty acid biosynthesis. It is likely that in CR rats BAT functions as an energy reservoir system in the form of TG (Fig. 7B). Thus, CR-associated functional alteration of mitochondria significantly differs between WAT and BAT, while it is likely that CR activates fatty acid biosynthesis and the metabolic process involving pyruvate, citrate, oxaloacetate and malate in both WAT and BAT. This metabolic process is known as pyruvate/malate cycling (Salway, 1999) or pyruvate/citrate cycling (Guay et al., 2007; Jensen et al., 2008). NADPH, which is generated by the pentose phosphate pathway and the chemical reaction catalyzed by malic enzyme (MAOX) in pyruvate/malate cycling, is a pivotal coenzyme for fatty acid biosynthesis. When energy supply is not sufficient, such as in CR animals, glucose is converted to pyruvate in the glycolytic pathway and does not mobilize to the pentose phosphate pathway. Therefore, we believe that in CR animals NADPH, which is vital for fatty acid biosynthesis, is predominantly generated by pyruvate/malate cycling. ACLY and MAOX, which are up-regulated by CR in both WAT and BAT, are involved in this cycle. Recently, it has been reported that certain

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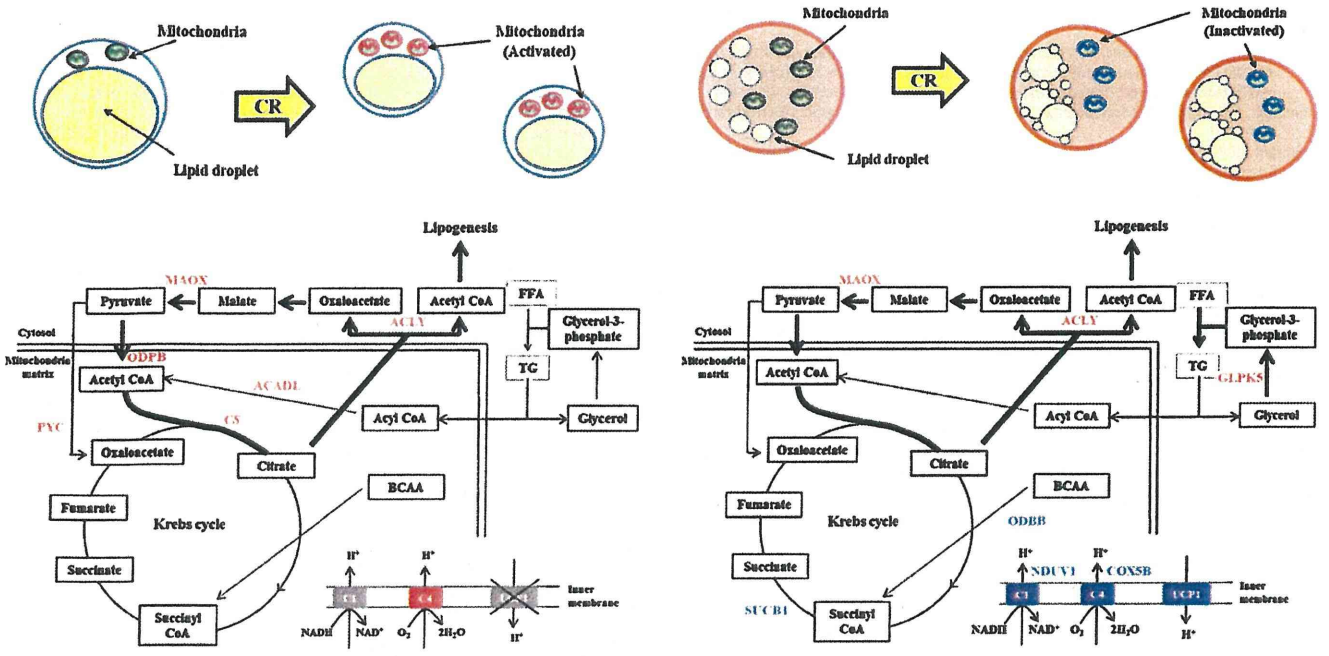


**Fig. 7.** Schematic diagram of the differential responses of WAT and BAT to CR based on the present study. In WAT, CR activates various mitochondrial functions including the Krebs cycle and the electron transport chain. It also accelerates fatty acid biosynthesis. It is likely that in CR rats WAT functions as an energy transducer from glucose to more energy-dense lipids and not as an energy storage system (A). In BAT, CR does not activate mitochondrial functions, but activates fatty acid biosynthesis. It is likely that in CR rats BAT functions as an energy reservoir system in the form of TG (B). CR-associated functional alterations of mitochondria significantly differ between WAT and BAT. However, it is likely that CR activates fatty acid biosynthesis and pyruvate/citrate cycling in both WAT and BAT. Expression of genes or proteins and enzymatic activities that were up-regulated by CR are indicated by red letters, and those down-regulated by CR are indicated by blue letters. ACADL: long-chain specific acyl-CoA dehydrogenase, mitochondrial; ACLY: ATP-citrate synthase; COX4: cytochrome c oxidase 4; COX5B: cytochrome c oxidase subunit 5B; CS: citrate synthase; FFA: free fatty acid; GLPK5: glycerol kinase 5; MAOX: NADP-dependent malic enzyme; NDUV1: NADH dehydrogenase flavoprotein 1, mitochondrial; ODBB: 2-oxoisovalerate subunit beta; ODPB: pyruvate dehydrogenase E1 component subunit beta, mitochondrial; PYC: pyruvate carboxylase, mitochondrial; SUCB1: succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial; TG: triglyceride; UCP1: uncoupling protein 1.

enzymes including ODPB and malate dehydrogenase, which are involved in pyruvate/malate cycling, are important regulators of lifespan in yeast (Easlon et al., 2007, 2008). Therefore, pyruvate/malate cycling may be a novel key regulator of the anti-aging and pro-longevity effects of CR.

Based on our data, we conclude that CR activates *de novo* fatty acid biosynthesis in both WAT and BAT. In contrast, CR enhances mitochondrial function in WAT but does not in BAT. The remodeling of both WAT and BAT, which is characterized by effective energy utilization, may promote beneficial actions associated with CR.

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# Phase II Trial of Preoperative Chemotherapy for Breast Cancer: Japan Breast Cancer Research Network (JBCRN)-02 Trial

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**Abstract.** *Background: Neoadjuvant chemotherapy (NAC) is one of the main strategies for patients with locally advanced breast cancer. In our previous study, biological markers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2 were essential predictors of the effectiveness of NAC to help individualize treatment. This study examined the effect of NAC on the disease-free survival (DFS) of breast cancer patients. Furthermore, the study was expanded by adding Ki-67 as a biological marker, and examined the correlation between Ki-67 and the prognosis. Patients and Methods: Between September 2005 and September 2007, 43 patients with breast cancer received NAC and surgery. Four cycles of DC (doxorubicin: 60 mg/m$^2$, and cyclophosphamide: 500 mg/m$^2$) were administered intravenously (i.v.) on day 1 every 21 days, followed by 12 cycles of paclitaxel i.v. (80 mg/m$^2$) every 7 days, prior to surgery. The primary endpoint was the pathological complete response (pCR) rate and the secondary endpoint was DFS; the pCR rate was estimated for each groups stratified by the presence or absence of different factors (PcR, ER/PgR, and Ki-67). Results: The clinical response (cCR+cPR) rate was 81.0%, and the pCR rate was 25.6%. The pCR rate was 75, 50, 9 and 0% in HER2$^+$/ER$^-$, HER2$^+$/ER$^+$, HER2$^-$/ER$^-$, and HER2$^-$/ER$^+$ patients, respectively. The 4-year DFS rate was estimated at 78% for all patients. The HER2 status was an independent predictor of pathological complete response (pCR). The DFS rate of patients with lower Ki-67 values (<15%) was higher than that of patients with higher Ki-67 values (≥15%). The treatment-related adverse events were manageable: the majority were mild, but five patients experienced grade 3 (neutropenia and sensory neuropathy) adverse events. Conclusion: DC followed by weekly paclitaxel is an active and manageable preoperative regimen for breast cancer patients. HER2 overexpression may be a good predictive marker of pCR, and the Ki-67 value after NAC may be a prognostic factor for DFS.*

Neoadjuvant chemotherapy (NAC) has emerged as a promising step forward in the management of locally advanced breast cancer. When administered before surgery, chemotherapy may induce tumor shrinkage, facilitate surgery, and increase the breast-conserving surgery rate (1-3).

The National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-27 demonstrated that compared to preoperative DC alone, the addition of sequential docetaxel doubled the pathological complete response (pCR) rate, increased the clinical complete response (cCR) rate, and increased the proportion of patients with negative axillary nodes (3-5). Some studies demonstrated that patients with pCR to chemotherapy had a good prognosis (1-5). Therefore the pathological response is an important prognostic parameter that can be used as a surrogate parameter for clinical outcomes. Furthermore, preoperative systemic therapy administering molecular targeted therapies, such as trastuzumab (Herceptin), and new hormone blockers, such as aromatase inhibitors, have been added to these regimens for the past 10 years (6). However pathological response cannot be accurately predicted.

In our previous study, biological markers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2 were essential predictors of the effectiveness of NAC to help individualize treatment (7). This study examined the effect of NAC on the disease-free survival (DFS) of breast cancer

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*Key Words:* Sequential therapy, adjuvant chemotherapy, breast cancer.

Table I. *Response criteria used in the present study.*

| | |
|---|---|
| Grade 0 (negative) | Almost no changes in post-treatment cancer cells. |
| Grade 1 (slight) | |
| 1a (mild) | Slight changes observed in cancer cells regardless of lesion size. |
| | Significant changes observed in <1/3 of cancer cells. |
| 1b (moderate) | Significant changes observed in 1/3 to <2/3 of cancer cells. |
| Grade 2 (significant) | Significant changes observed in approximately ≥2/3 of cancer cells. |
| Grade 3 (complete) | All cancer cells necrotize or disappear, replaced with granuloma-like tissues or focal fibrosis. |

patients. In addition, we expanded the study by adding Ki-67 as a biological marker. We conducted a multicenter prospective neoadjuvant trial with four cycles of doxorubicin and cyclophosphamide (DC) followed by twelve cycles of paclitaxel for breast cancer patients to investigate the relationship between pathological effect and survival. Clinical response, the rate of breast-conserving surgery (BCS), some factors, and safety were also evaluated.

## Patients and Methods

This multicenter, open-label, single-arm, phase II study was conducted in women aged 20 to 69 years with previously untreated unilateral carcinoma of the breast (T2-3, N0-1, M0). Patients with bilateral, locally advanced, or metastatic disease were excluded. Other eligibility criteria included: Eastern Cooperative Oncology Group performance status 0 to 1; adequate bone marrow reserve (absolute neutrophil count (ANC) >2,000/mm$^3$, platelet count >100,000/mm$^3$), and adequate renal (serum creatinine <1.5 times upper normal limit) and hepatic function (total bilirubin <2 times upper normal limit); left ventricular ejection fraction (LVEF) within normal limits based on echocardiographic (ECG) assessment. Patients were excluded from the study if they had any history of another neoplasm. All patients gave written informed consent before their participation in the trial. The study was conducted in accordance with the Declaration of Helsinki. The protocol was reviewed and approved by the Institutional Review Boards at all participating centers, and written informed consent was obtained from all patients prior to the study.

Four cycles of DC (doxorubicin: 60 mg/m$^2$ and cyclophosphamide: 500 mg/m$^2$) administered intravenously (*i.v.*) on day 1 every 21 days were followed by 12 cycles of paclitaxel *i.v.* (80 mg/m$^2$) every 7 days, prior to surgery. Treatment was continued in the absence of unacceptable toxicity. Premedication 30 min prior to paclitaxel administration consisted of *i.v.* ranitidine (50 mg), and *i.v.* dexamethasone (20 mg), and oral diphenhydramine (50 mg). Prophylactic hematologic growth factor support was prohibited before the second course of treatment.

The disease status was confirmed by physical examination, mammography, and breast ultrasonography and a core or fine-needle biopsy for histopathological diagnosis. During treatment, white blood cell count was repeated weekly. Biochemistry tests were performed after courses 2 and 4, and cardiac monitoring comprised an ECG after course 4 and LVEF measurement after courses 2 and 4, or after study discontinuation. Adverse events were evaluated according to CTC grades.

Treatment was to be postponed for a maximum of 2 weeks for severe toxicity. If toxicity did not improve during this period, chemotherapy was discontinued and surgery was recommended. Dose reductions of doxorubicin from 60 to 40 mg/m$^2$, cyclophosphamide from 600 to 400 mg/m$^2$, and paclitaxel from 80 to 60 mg/m$^2$ were permitted in cases of febrile neutropenia and grade 3 or 4 non-hematological toxicities except for nausea, vomiting, and fatigue. Following chemotherapy and clinical assessment of the response, patients underwent surgery. If the tumor was too large or invasive for BCS, modified radical mastectomy was recommended. Sentinel lymph node biopsy was not performed to confirm the disease stage.

*Assessment of response to therapy.* A physical examination was performed and the performance status was assessed on day 1 of each course. Tumor assessment involved a physical examination before, during, and after every course and breast ultrasonography after 4 courses of DC regimen; the appearance of any new lesion was documented. The primary endpoint was to determine the rate of pCR induced by primary chemotherapy and assessment of the pathological response as an independent predictor of DFS. The pathological response was classified according to the criteria in Table I.

The clinical response of bidimensionally measurable and assessable disease was classified as a complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to WHO criteria. CR was defined as the disappearance of all clinical evidence of the tumor; PR was defined as a 50% or more reduction in the sum of the products of measured lesions, or an estimated decrease in the tumor size of at least 50%, without the appearance of new lesions; SD was defined as a decrease in the lesion size of less than 50% for the sum of the products of measured lesions, or an estimated decrease of less than 50% and increase of less than 25%, without the appearance of new lesions. Any measured or estimated increase greater than 25% or the appearance of new lesions was defined as PD. The clinical response was defined as the sum of CRs and PRs. Surgery was to be performed less than 4 weeks after the last chemotherapy course.

Where possible, breast-conserving methods were carried out, taking into account the residual tumor size after chemotherapy, and esthetics. After a complete clinical response to chemotherapy, when feasible, a wide surgical excision was performed to remove the tumor with free margins without deforming the breast. Postoperative irradiation was delivered to the breast and regional lymph nodes according to local practices. After chemotherapy, a mastectomy was carried out if the initial multifocal disease could not be removed by a single wide excision or if an extensive area of radiological microcalcifications did not regress with chemotherapy (even though a cCR had been achieved). Hormonal treatment with tamoxifen was given to all patients with ER$^+$ tumors, and any additional chemotherapy was administered at the discretion of the investigator. Follow-up was performed every 4 months for the first 2 years, thereafter every 6 months, and once a year after 5 years. A total of 43 assessable patients were enrolled in the study.

Table II. *Patient characteristics, n (%).*

| | | |
|---|---|---|
| Stage | 1 | 2 (4.7%) |
| | 2a | 10 (23.3%) |
| | 2b | 17 (39.5%) |
| | 3a | 6 (14.0%) |
| | 3b | 3 (7.0%) |
| | 3c | 5 (11.6%) |
| Tumor size (mm) | <20 | 4 (9.3%) |
| | 20 + | 39 (90.7%) |
| ER | Positive | 28 (65.1%) |
| | Negative | 15 (34.9%) |
| PgR | Positive | 25 (58.1%) |
| | Negative | 18 (41.9%) |
| HER2 | 0 | 19 (44.2%) |
| | 1+ | 5 (11.6%) |
| | 2+ | 5 (11.6%) |
| | 3+ | 14 (32.6%) |
| Pathological grade | 1 | 15 (34.9%) |
| | 2 | 24 (55.8%) |
| | 3 | 3 (7.0%) |
| | Unknown | 1 (2.3%) |
| Lymph-node status | 0 | 27 (62.8%) |
| | 1-3 | 9 (20.9%) |
| | 4+ | 5 (11.6%) |
| | Unknown | 2 (4.7%) |

Table III. *Prediction of pCR (G3) by logistic regression.*

| Factors | % pCR | Statistics | Univariate analysis | Multivariate analysis |
|---|---|---|---|---|
| Age | | | | |
| <50 years | 23.8% (5/21) | OR | 1.19 | 1.80 |
| ≥50 years | 27.3% (6/22) | *P*-value | 1.000 | 1.000 |
| Tumor size | | | | |
| <30 mm | 0.0% (0/7) | OR | 3.92 | 3.82 |
| ≥30 mm | 30.6% (11/36) | *P*-value | 0.209 | 0.288 |
| ER | | | | |
| – | 40.0% (6/15) | OR | 2.98 | 1.19 |
| + | 17.9% (5/28) | *P*-value | 0.225 | 1.000 |
| PgR | | | | |
| – | 38.9% (7/18) | OR | 3.24 | 0.93 |
| + | 16.0% (4/25) | *P*-value | 0.180 | 1.000 |
| HER2 | | | | |
| 2+ | 6.9% (2/29) | OR | 21.72 | 21.07 |
| 3+ | 64.3% (9/14) | *P*-value | <0.001 | 0.003 |
| Clinical response | | | | |
| SD+PD | 11.1% (1/9) | OR | 3.26 | 3.17 |
| CR+PR | 29.4% (10/34) | *P*-value | 0.510 | 0.762 |

CR: Complete response; PR: partial response; SD: stable disease; PD: progressive disease; OR: odds ratio.

*Histopathological examination.* Pretreatment diagnosis was established by our pathologists using samples from core needle biopsy. The items investigated were the presence or absence of lymph node metastasis, nuclear grade, ER/PgR status, and HER2. Recent data suggest that several biological markers, especially Ki-67, may have the potential to predict the effectiveness of NAC with anthracycline and taxane. Therefore, we performed a post-hoc analysis of outcomes according to Ki-67. Immunostaining of ER, PgR, Ki-67, and HER2 was conducted as previously described (8). The positive cell rates for ER/PgR were determined by Immunohistochemistry. An assessment value of 10% or higher was rated as positive. Proliferative activity was determined by immunostaining for Ki-67 antibody (Dako, Tokyo, Japan). The fraction of proliferating cells was based on a count of at least 500 tumor cells. The Ki-67 values were expressed as the percentage of positive cells in each case.

*Statistical analysis.* The primary endpoint was the pCR rate of the treatment. Pathological response grades were stratified by tumor and nodal staging, patient age, and clinical response. Secondary endpoints included predictors for pCR, DFS, the rate of breast-conserving surgery, and safety. A 10-30% pCR rate was reported based on histopathology in preoperative anthracycline plus taxane (PTX) chemotherapy regimens. The required number of patients was calculated as 41, using a 25% expected efficacy rate, 10% threshold efficacy rate, two-sided alpha level of 0.05, and 80% power for the statistical analysis of the primary endpoint for this sequential combination chemotherapy. Analyses were performed with JMP (version 9; SAS Institute Inc., Tokyo, Japan).

## Results

*Patient characteristics.* Between April 2004 and March 2007, 43 patients were prospectively enrolled. The characteristics of the study population are presented in Table II. The median age was 50 (range: 20-69) years. The majority of patients had T2 tumors.

*Efficacy of NAC.* The patients were evaluable regarding their response and toxicity. Clinical responses were rated as cCR in 9 patients (22%), cPR in 25 patients (59%), and cSD in 9 patients (19%). The pCR was seen in 25.6%. Breast-conserving surgery was achieved in 58% of all 43 patients. Furthermore, multiple logistic regression analysis was performed to examine factors including menopausal status, tumor size, ER status, PgR status, HER2 status, and clinical response (Table III). Multivariate analysis showed that the HER2 status was an independent predictive factor of pCR. The pCR rates stratified by HER2 and ER are shown in Figure 1. The pCR rate was 75%, 50%, 9% and 0 % in $HER2^+/ER^-$, $HER2^+/ER^+$, $HER2^-/ER^-$, and $HER2^-/ER^+$ patients, respectively.

The estimated 4-year DFS was 78% for all patients. Patients who achieved pCR did not show an improved DFS compared to those without pCR (log-rank test, $p<0.05$, Figure 2). Because of evidence that Ki-67 may be useful to evaluate the neoadjuvant setting (8, 9), we evaluated the influence of the Ki-67 status and pCR. This analysis should be regarded as exploratory, because it was not prespecified. As a result, the DFS rate of patients with lower (<15%) Ki-67 values was higher than that of patients with higher (≥15%) Ki-67 values.

The toxicities were manageable and the safety profile is summarized in Table IV. Dose reduction and interuptation due

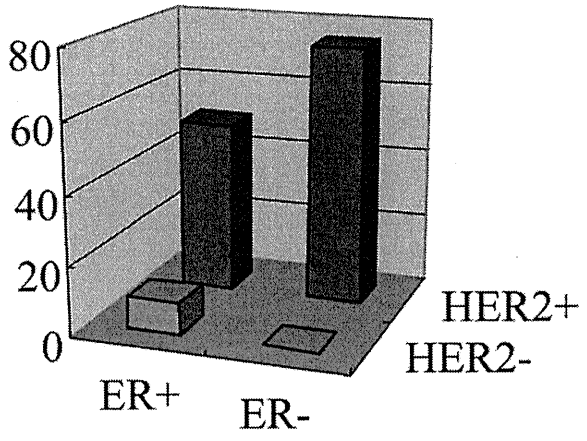


Figure 1. *Relationship between pCR and HER2/ER status.*

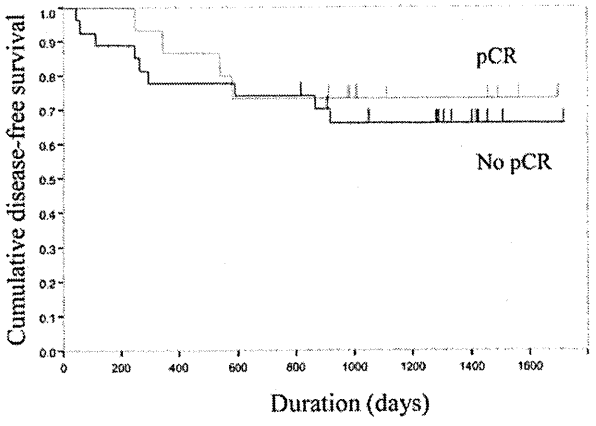


Figure 2. *Relationship of pCR and non-pCR to disease-free survival.*

to toxicities did not occur during treatment. The most common toxicity was nausea, which was observed in 62.8% of patients during DC treatment and 33% of patients during paclitaxel treatment. Grade 3-4 nausea was not seen in either treatment. Grade 3 neutropenia was reported in 2.3% and 7.1% of patients during treatment with DC and paclitaxel, respectively.

## Discussion

Our study demonstrates that DC followed by paclitaxel is a promising NAC regimen for patients with breast cancer not amenable to conservative surgery. In other studies, the regimen of three cycles of 5-fluorouracil plus epirubicin plus cyclophosphamide followed by three cycles of docetaxel at 100 mg/m$^2$ led to the favorable result of an 18% risk reduction in DFS and 27% risk reduction in overall survival. However, in Japan, the standard dose of docetaxel is 75 mg/m$^2$. Therefore, we selected DC followed by weekly paclitaxel, and showed that the actual 4-year DFS rate of 78% was similar to the results of other studies (1-5). Unfortunately, there was no significant improvement in DFS regardless of the existence of pCR, possibly because this was not a large study. However, the DFS rate of patients with lower Ki-67 values (<15%) was higher than that of patients with higher values (≥15%).

Regarding toxicity, there were no severe toxic effects as compared with other recent studies (1-5). In terms of the incidence of febrile neutropenia, it was lower than that of other studies. (1-5). This confirms that DC followed by weekly paclitaxel as the neoadjuvant setting is appropriate for Japanese women.

In addition, we investigated ER, PgR, HER2, and Ki-67. We found that the pCR rate was the highest in patients who were ER$^-$/HER2$^+$. pCR was significantly associated with HER2 positivity based on multivariate analysis. Furthermore, in the present study, a higher pCR was often found in patients with tumors with a higher Ki-67 value, and there was no pathological responder in cases with Ki-67 <15% (data not shown). Regarding breast cancer subtypes, Ki-67 values were higher in patients with triple-negative tumors (10-13). These tumors respond more frequently to a neoadjuvant setting. On the other hand, ER$^+$ and/or PgR$^+$ tumors had lower Ki-67 values (10-13). These tumors respond more frequently to endocrine therapy. Therefore, clarifying the proliferative activity may be important for the treatment of breast cancer.

HER2 overexpression was suggested to be a predictor of the sensitivity to anthracycline chemotherapy (12). Indeed, in this study, HER2 was the only predictive factor for pCR. However, in the present study, trastuzumab was not administered to patients with HER2-overexpressing tumors because its use in such a setting has not yet been approved in

Table IV. *Treatment-related toxicities reported by patients in the study.*

| | DC (N=43) | | Paclitaxel (N=42) | |
|---|---|---|---|---|
| Toxicity | All grades | Grade 3+ | All grades | Grade 3+ |
| Neutropenia | 17 (39.5%) | 1 (2.3%) | 17 (40.5%) | 3 (7.1%) |
| Nausea | 27 (62.8%) | 0 (0.0%) | 14 (33.3%) | 0 (0.0%) |
| Vomiting | 19 (44.2%) | 0 (0.0%) | 7 (16.7%) | 0 (0.0%) |
| Hair loss | 19 (44.2%) | 0 (0.0%) | 7 (16.7%) | 0 (0.0%) |
| Stomatitis | 8 (18.6%) | 0 (0.0%) | 1 (2.4%) | 0 (0.0%) |
| Peripheral neuropathy | 4 (9.3%) | 0 (0.0%) | 24 (57.1%) | 2 (4.8%) |
| Subungual bleeding | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| Hand–foot syndrome | 0 (0.0%) | 0 (0.0%) | 1 (2.4%) | 0 (0.0%) |
| Diarrhea | 0 (0.0%) | 0 (0.0%) | 2 (4.8%) | 0 (0.0%) |

Japan. Recently, trastuzumab was found to significantly improve the prognosis and response to chemotherapy in such patients; the pCR rate was significantly higher in patients who were treated with trastuzumab (15-17). The relationship between HER2 overexpression and the response to chemotherapy with trastuzumab needs future investigation.

In conclusion, DC followed by weekly paclitaxel is safe, feasible, and effective as a preoperative adjuvant chemotherapy for Japanese women with breast cancer.

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