Dewaxed Brown Rice Contains a Significant Amount of Lipopolysaccharide Pointing to Macrophage Activation *via* TLRs

HIROYUKI INAGAWA[1,2,3,4], TOSHIYUKI SAIKA[5], TAKASHI NISIZAWA[3], CHIE KOHCHI[1,2,3], MAYA UENOBE[6] and GEN-ICHIRO SOMA[1,2,3,4]

[1]*Control of Innate Immunity, Technology Research Association, Kagawa, Japan;*
[2]*Faculty of Medicine, Kagawa University, Kagawa, Japan;*
[3]*Macrophi Inc., Kagawa, Japan;*
[4]*Research Institute for Healthy Living, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan;*
[5]*Toyo Rice Corp., Wakayama, Japan;*
[6]*Nagoya Keizai University, Aichi, Japan*

Abstract. *Background/Aim: Oral ingestion of lipopolysaccharide (LPS) has been shown to be effective in diseases' prevention. Brown rice contains large amounts of LPS not actively consumed because of bad taste. Recently, a new type of brown rice with its wax layer removed has been produced. In this report, we measured the LPS content of this dewaxed rice and evaluated the function of innate immune activation on macrophages. Materials and Methods: Dewaxed brown rice and polished rice were prepared using the Saika-style rice polishing process. LPS content extracted using hot water from this sample was evaluated by the Limulus reaction and the activation of macrophage RAW246.7 cells was evaluated by nitric oxide (NO) production. In addition, toll-like receptors (TLRs) 2-, 4- and 9-induced human embryonic kidney (HEK) 293 cells were used for the confirmation of the activated pathway. Results: Mean LPS content in the 15 types of dewaxed brown rice was found to be 6.4±2.6 μg/g, while that of brown rice was 10.9±4.3 μg/g. The extract of dewaxed brown rice induced significant amounts of NO by RAW246.7 cells, while production was reduced to 1/6 by adding polymyxin B. The macrophage activating effect of dewaxed brown rice was 79- and 51-times higher than that of polished rice in TLR4- and 2-induced HEK 293 cells. Conclusion: LPS content in dewaxed brown rice was found to be able to activate macrophages. This rice activated macrophages mainly via the TLR4 and, to a lesser extent, TLR2 pathways. It is suggested that dewaxed brown rice can be considered effective in allergy and cancer prevention.*

Correspondence to: Dr. Hiroyuki Inagawa, Faculty of Medicine, Kagawa University, 2217-16 FROM-KAGAWA Bio Lab., Hayashi-cho, Takamatsu-shi, Kagawa-ken, 761-0301, Japan. Tel/Fax: +81 8139203, e-mail: PXS07205@nifty.ne.jp

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LPS is a molecule derived from the outer membrane of Gram-negative bacteria known to activate macrophages and both regulatory T (1) and intestinal epithelial cells (2) *via* the toll-like receptors (TLR) 4 pathway and regulate innate immunity. We have previously reported that LPS enhances the phagocytic capacity, expressed as a priming effect (enhancement of cytokine production by triggering agents) in mice after oral administration (3, 4), improves the effects of cancer chemotherapeutic agents (5) and is effective in preventing allergic diseases (6, 7).

It has been demonstrated that many edible plants contain LPS (0.0001 to 100 μg/g of dry plant weight) (8). Plant LPS is considered to be exclusively derived from Gram-negative bacteria that are symbiotic to plants (9). These symbiotic bacteria have been reported to be involved in nitrogen fixation, inorganic phosphorus solubilization and infection protection (10-12). This LPS, when orally ingested, has been shown to be involved in maintaining health. Even Chinese herbal medicines comprise plants containing LPS (8), with an example being the Juzentaihoto (Kampo) that has been reported to activate macrophage due to its LPS content (13). Therefore, it is considered that orally-ingested LPS-containing daily food is a promising cancer prevention aliment.

During the course of edible plant screening, we discovered that brown rice contains high levels of LPS. Brown rice consists of the outer layers of pericarp, seed-coat and nucellus; the germ or embryo; and the endosperm. Rice polishing is a process in which the bran layer covering the

surface of the brown rice (mainly pericarp and aleurone layers) is removed and only the albumen part is retained. Polished rice is the rice in which the bran layer and germ are removed. In Asian countries, polished rice is a staple food that is consumed more than any other food. Especially, brown rice is considered to be a food with a better nutritional composition in comparison with polished rice because the bran layer and germ contain vitamin E, vitamin B1, dietary fiber, maltooligosaccharides, *etc*. (14). As already described, brown rice is believed to be a promising functional food. However, consumption of brown rice is generally limited because of its poor digestion and unpleasant texture by the hard bran layer (15). The fat content in the outermost layer (wax bran layer) repels water; water absorption of brown rice is poor. As a consequence, gelatinization is incomplete if brown rice is cooked in a rice cooker designed for cooking white rice. Rice milling technology, which removes the wax bran layer from brown rice while retaining the nutrients and improving the taste, has been developed (Saika-style rice polishing process). Removal of the wax bran layer has solved the issue of repelling water from the surface and preventing water absorption into the rice, thereby significantly improving its taste and digestion.

Focusing on LPS as the functional component of rice, we have previously reported that LPS contained in orally-consumed rice has anti-allergic effects *via* macrophage activation (16). Although brown rice contains more LPS than polished rice, LPS has not been evaluated in the dewaxed rice. The purpose of this study was to clarify the LPS content and macrophage activation capability of dewaxed brown rice and to analyze the involvement of TLRs in macrophage activation.

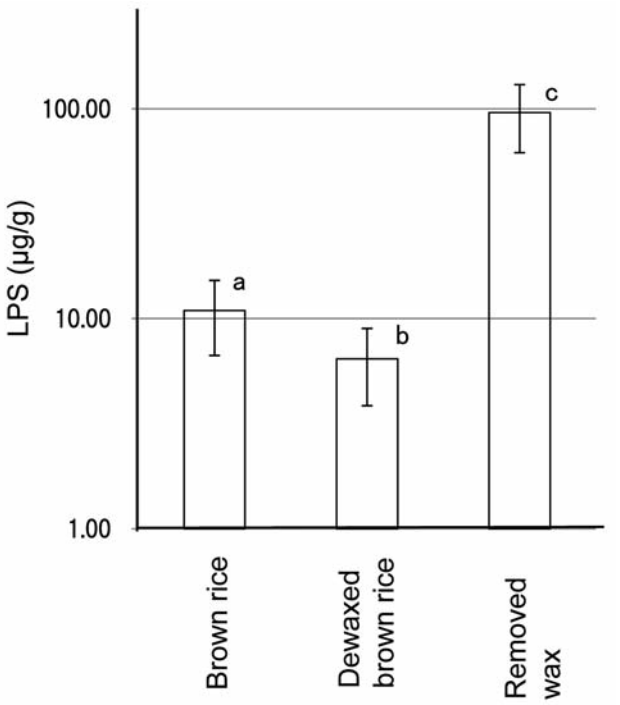

Figure 1. *Measurement of LPS content in rice samples. The amount of LPS was measured from prepared rice sample using the Limulus reaction. Columns and bars indicate means and standard deviations (n=15), respectively. The letters a, b, and c indicate statistical differences between means (Student t-test, p<0.05).*

Materials and Methods

Preparation of rice samples. A total of 15 samples from the 2014 crop were used (two samples each from the Aomori, Iwate, Shiga and Oita Prefectures; three samples from the Tottori Prefecture; and one sample each from the Fukui, Niigata, Yamagata and Ishikawa Prefectures). Each batch of brown rice was dewaxed and polished rice was prepared using the Saika-style rice polishing process in Toyo Rice Corp. Thereafter, it was treated by the rinse-free process. For LPS measurements, one gram of each sample was added up to 10 ml distilled water and heat-treated (90˚C for 20 min). The samples were subsequently sonicated for 15 min and centrifuged for 30 min at 3,500 rpm. The collected supernatants were used for LPS experiments (hereinafter 100 mg/ml rice extract of each sample).

LPS content estimation by Limulus amoebocyte gelation assay. LPS amount in samples was assayed by the kinetic turbidimetric method. All samples were diluted 10-fold with pyrogen-free distilled water. A 0.1 ml sample of test fluid was added to 0.1 ml of LAL-ES in a glass tube (Limulus ES-II test; Wako Pure Chemical Industries Ltd., Osaka, Japan). After vortex mixing for a few seconds, the gelation time was measured with a Toxinometer ET-201 (Wako Pure Chemical Industries Ltd.) and the specific activity was calculated with an LS Toximaster (Wako Pure Chemical Industries Ltd.), a data acquisition program for the Toxinometer.

Nitric oxide (NO) production by murine macrophages. Cells from the mouse macrophage/monocyte cell line RAW264.7 (TIB-71; ATCC, Manassas, VA, USA) were plated at 8×10^5 cells/ml and exposed to various concentrations of rice extracts at 37˚C in a 5% CO_2 incubator. Twenty-four hours after exposure, the supernatant was analyzed for NO release. NO activity was monitored as nitrite (NO_2^-) release in culture media after 24 h of incubation, using Griess reagent. One hundred μl of Griess reagent was added to 100 μl of diluted culture media in the wells of microtiter plates. After incubation at room temperature for 10 min, the absorbance at 570 nm was determined by means of an automated microplate reader (MTP-32; Corona Electric Co., Ibaraki, Japan). The NO assay was repeated two times. Polymyxin B (Sigma-Aldrich, St. Louis, MO, USA) was added to each culture at a final concentration of 10 μg/ml.

Identification of the receptor for the detection of macrophage activating components. To elucidate the Toll-like receptor (TLR) activation pathway of the test samples, human embryonic kidney (HEK) 293 cells transfected with TLR2, TRL4/MD2/CD14 or TLR9 (Invitrogen, Waltham, MA, USA) were used. HEK293 cells are characterized to express interleukin (IL)-8 when activated *via* the transfected TLR pathway. Cells were adjusted to contain 4×10^5

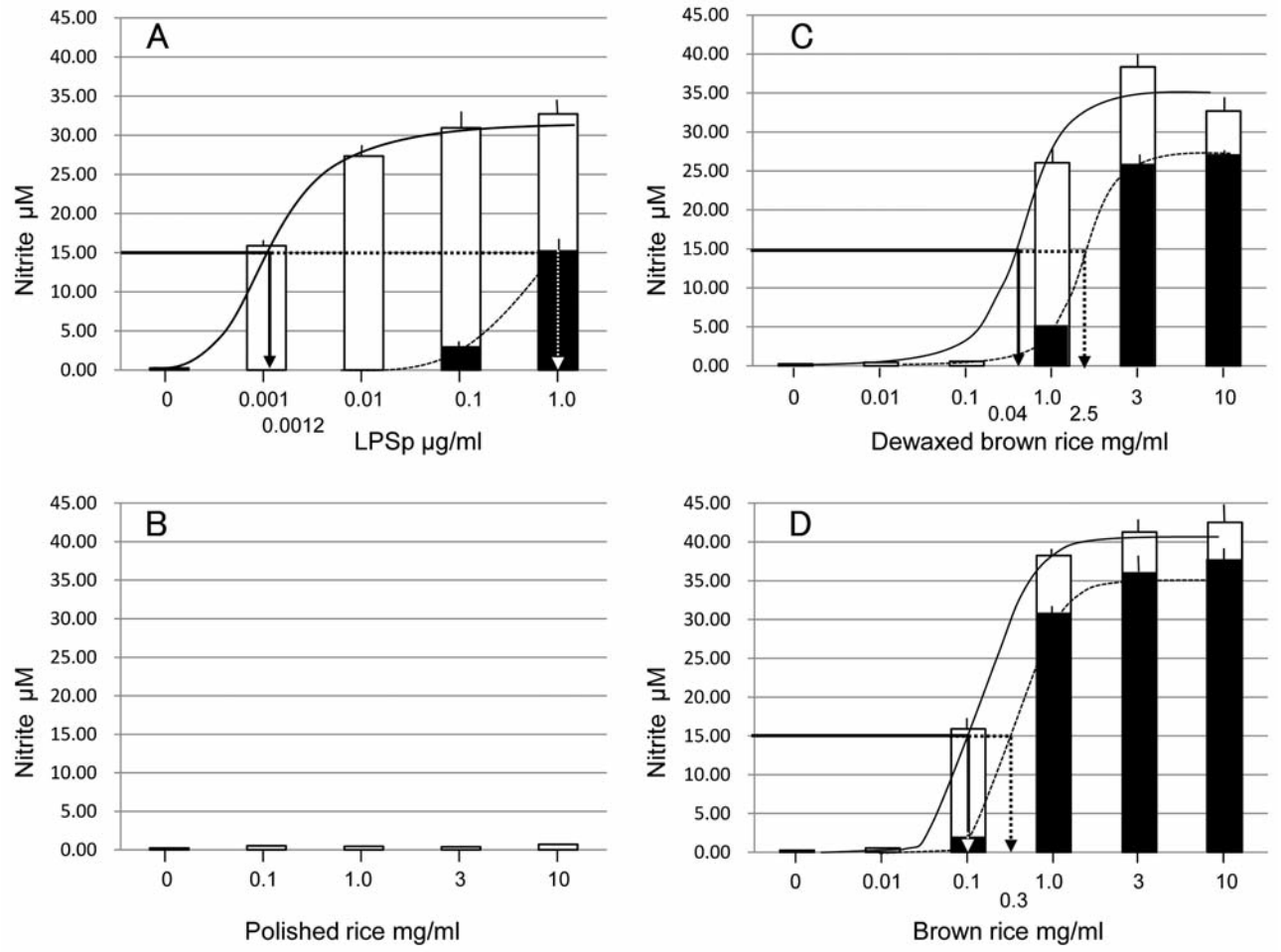

Figure 2. *NO production and polymyxin B effect by rice samples on macrophage cell culture. The rice samples were added to murine macrophage cells J774 and NO_2 content was measured in the supernatant after 24 h of culture by the Griess reaction. A: Purified LPS derived from P. agglomerans. B: Extract from polished rice. C: Extract from dewaxed brown rice. White column does not contain polymyxin B, while black column contains 10 μg/ml of polymyxin B. Arrows indicate the estimated concentration (underlined values) of rice sample by producing 15 μM of nitrite. Columns and bars indicate means and standard deviations (n=6), respectively.*

cells/ml, then 100 μl of cells were added per well to a 96-well flat bottom plate and cultured for 24 h at 37˚C. After culturing, cells were washed with 100 μl of fresh culture solution. Test solutions were added into each well at 100 μl and cultured for 24 h. After culturing, ELISA measurements of IL-8 (Biolegend, San Diego, CA, USA) were conducted using culture supernatants from each well.

Statistical analysis. Statistical analysis was performed using Excel 2008 ver.1.07 (SSRI, Tokyo, Japan).

Results

LPS measurement in dewaxed brown rice. We obtained 15 strains of brown rice from nine Japanese rice production regions and prepared dewaxed brown rice samples. Samples prepared using brown rice and dewaxed brown rice by hot water extraction were used for LPS measurements. Results are shown in Figure 1. LPS content in brown rice and dewaxed brown rice was 10.6±4.3 μg/g and 6.4±2.7 μg/g, respectively. Approximately 40% of LPS was lost through dewaxing.

Measurement of nitric oxide (NO) production capacity of dewaxed brown rice samples. To confirm macrophage activation by dewaxed brown rice, we examined the dose response of dewaxed brown rice, brown rice and polished rice using NO production from RAW246.7 cells as an indicator. As shown in Figure 2, although the polished rice (LPS content: 0.037 μg/g) extracts with a maximum concentration of 10 mg/ml (10 mg of dry weight polished rice per 1 ml) could not produce detectable levels of NO (Figure

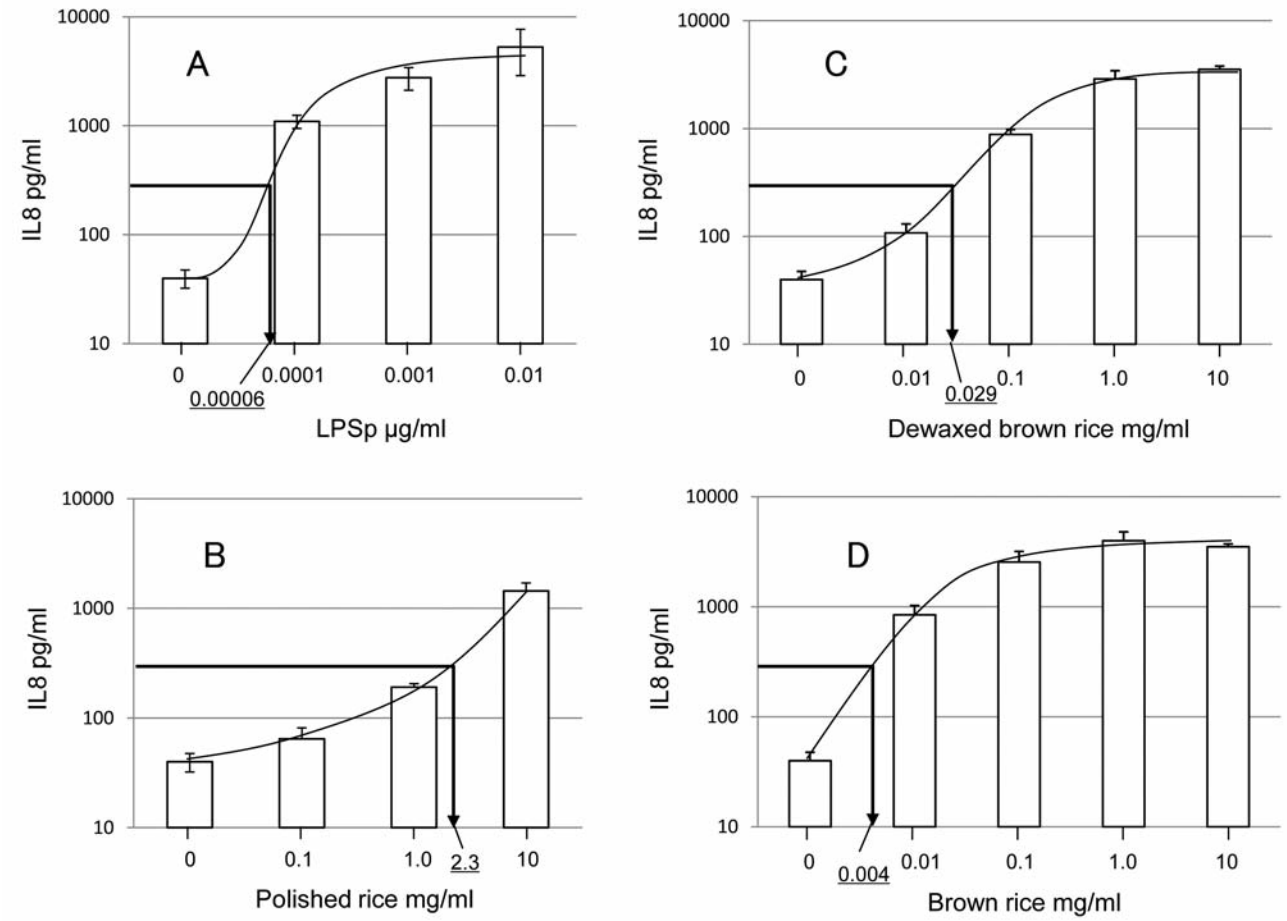

Figure 3. *Evaluation of TLR4 response of rice samples as function of IL-8 production. The rice samples were added to TLR4/MD2/CD14-expressing HEK 293 cells and the amount of IL-8 was measured in the supernatant using ELISA after 24 h of culture. A: Purified LPS derived from P. agglomerans. B: Extract from polished rice. C: Extract from dewaxed brown rice. Arrows indicate the estimated concentration (underlined values) of rice sample by producing 300 pg/ml of IL-8. Columns and bars indicate means and standard deviations (n=4), respectively.*

2B), samples from hot water extracts of dewaxed rice (LPS content: 2.6 μg/g) showed a 15 μM of nitrite production (ED15) even with a concentration of 0.04 mg/ml (Figure 2C), while brown rice (LPS content: 17 μg/g) showed 0.1mg/ml (Figure 2D).

To measure the ratio of LPS that is involved in macrophage activation by dewaxed brown rice, NO production using polymyxin B, which is known to inhibit LPS by binding to it, was concurrently evaluated. The black column in Figure 2 shows NO production upon addition of 10 μg/ml of polymyxin B. NO production of LPS from *Pantoea agglomerans* as a positive control was reduced to <1/1,000 by adding polymyxin B. On the other hand, NO production of dewaxed brown rice was reduced to 1/6 by adding polymyxin B. These results suggest that the main component of macrophage activation was contributed by LPS-containing dewaxed brown rice.

Confirmation test of receptors for macrophage activation caused by components in dewaxed brown rice. LPS, which is the main component in the dewaxed brown rice for macrophage activation (Figure 2), is derived from symbiotic Gram-negative bacteria. To evaluate the involvement of peptidoglycan and β-glucan, which have been predicted as macrophage activation components derived from other bacteria, the amount of secreted IL-8 was measured using hTLR2- and hTLR4- expressing HEK 293 cells.

We observed a dose-dependent increase of IL-8 from each rice sample in TRL4/Myeloid differentiation factor (MD) 2/cluster of differentiation (CD)14-expressing HEK 293 cells (Figure 3) and TLR2-expressing HEK 293 cells (Figure 4). For comparing the concentration of rice samples required for 300 pg/ml of IL-8 production in TRL4/MD2/CD14-HEK 293 cells, polished rice, dewaxed brown rice and brown rice showed a production of 2.3 mg/ml, 0.029 mg/ml and

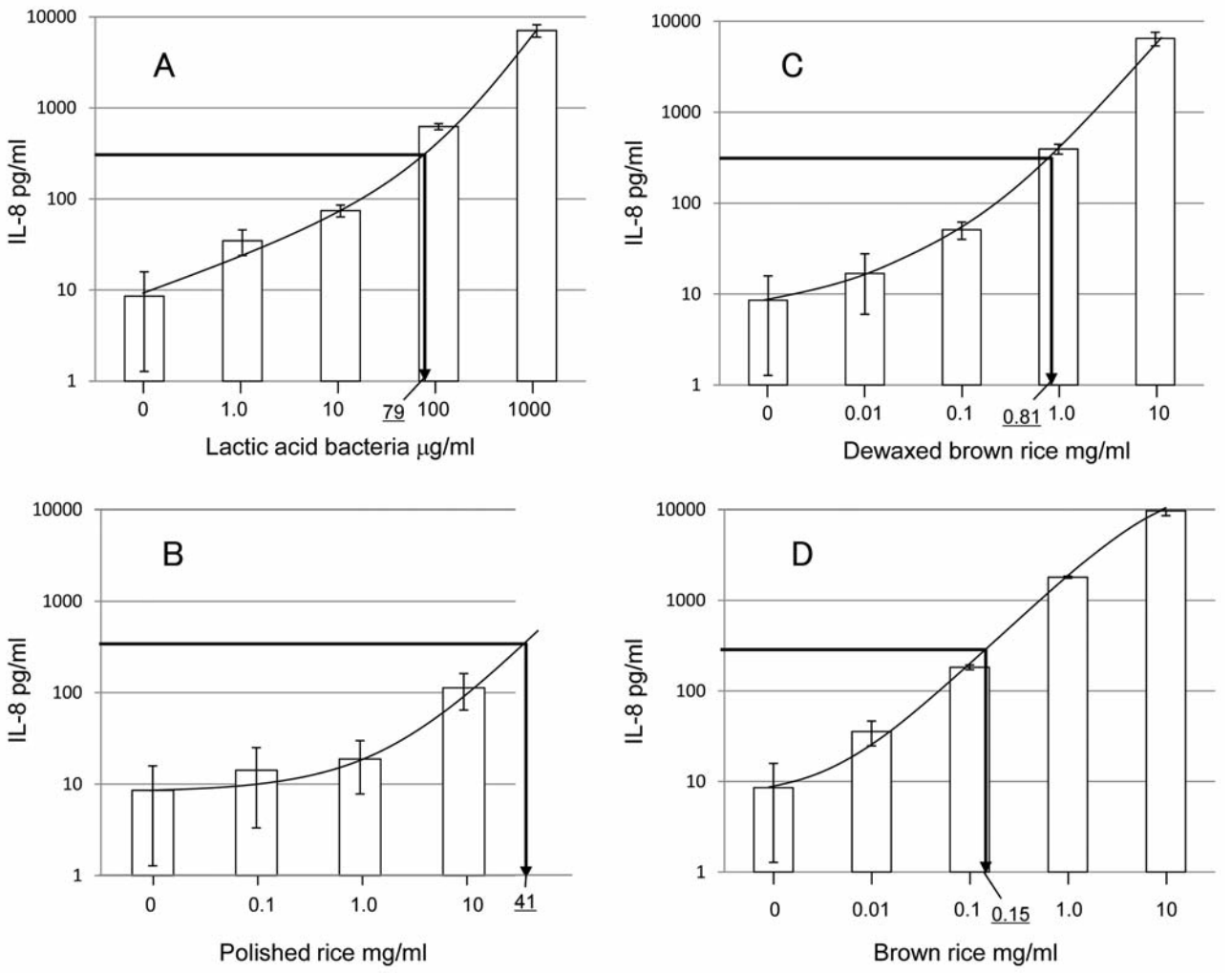

Figure 4. *Evaluation of TLR2 response of rice samples. The rice samples were added to TLR2-expressing HEK 293 cells and the amount of IL-8 was measured in the supernatant using ELISA after 24 h of culture. A: Purified LPS derived from P. agglomerans. B: Extract from polished rice. C: Extract from dewaxed brown rice. Arrows indicate the rice sample producing 300 pg/ml of IL-8. Arrows indicate the estimated concentration (underlined values) of rice sample producing 300 pg/ml of IL-8. Columns and bars indicate means and standard deviations (n=4), respectively.*

0.04 mg/ml, respectively (Figure 3). In TRL2-HEK 293 cells, polished rice, dewaxed brown rice and brown rice showed 41 mg/ml, 0.81 mg/ml and 0.15 mg/ml production, respectively (Figure 4). Thus, the macrophage-activating effect of dewaxed brown rice was 79 and 51 times higher than that of polished rice in TLR4- and 2-induced HEK 293 cells.

Discussion

We have previously reported that the subaleurone layer of the outer layer, in contact with the starch layer of rice, can activate macrophages, with the main component of this activation deriving from LPS (16). As shown in Figure 1, dewaxed brown rice contains an average of 6 μg/g of LPS (standard *Escherichia coli* LPS conversion). LPS derived from *P. agglomerans* was used as a positive control and produced 15 μM of nitrite after the addition of 1 ng/ml LPS. Thus, it is considered that nitic oxide production by hot water extraction of dewaxed brown rice was caused mainly by LPS.

LPS has been reported to be contained in various edible plants and herbal medicines (8). LPS contained in these plants is thought to be derived predominantly from symbiotic Gram-negative bacteria. Some, known as endophyte bacteria, enter plants, survive and enhance the host's ability to protect itself from infection. Since it is known that some symbiotic Gram-negative bacteria exist in rice (17) and wheat (18), it is conceived that people ingest LPS derived from bacteria on a daily basis.

Although it has been reported that plants have the potential of making lipid A of LPS (19), it is believed the LPS contained in agricultural products is derived from predominantly symbiotic bacteria. Thus, we predicted a large variation in LPS content in brown rice, which is not regulated by the number of symbiotic bacteria. However, the actual variations were smaller than predicted (Figure 1). This leads to the speculation that brown rice actively maintains symbiotic bacteria at a certain density.

Macrophages are the major phagocytes to process not only invading pathogens, such as intrusive bacteria and viruses, but also modified products and undesired substances that occur as a foreign substances in the body. Removal of these foreign objects plays a very important role in maintaining the health of living organisms and constitutes a central function of innate immunity (20). As shown in Figure 2, extracts of dewaxed brown rice induced significant amount of NO by the macrophage cell line employed. The observed macrophage activation effects caused by dewaxed brown rice decreased to approximately 1/6 by the addition of polymyxin B (Figure 2C). This suggests that the main component for macrophage activation caused by rice appears to be an LPS-like substance. Data from TLR4-, MD2- and CD14-expressing HEK 293 cells showed TRL4 to be a primary signal (Figure 3). On the other hand, it was also demonstrated that some components of macrophage activation were not completely inhibited by polymyxin B. The experiment, however, on HEK 293 cell cultures supports the existence of a TLR2 signal for macrophage activation (Figure 4).

We have demonstrated that oral administration of *Pantoe agglomerans* (plant symbiotic Gram-negative bacteria) LPS increased the phagocytic activity of peritoneal macrophages (4). Furthermore, it has been reported that LPS enhances the antitumor effect of cancer drugs (doxorubicin) in mice transplanted with melanoma (5). To date, research has shown that oral administration of LPS effectively decreases low-density lipoprotein (LDL) levels of individuals with high LDL (3) and prevents loss of bone density in postmenopausal women (6). As dewaxed brown rice contains an average 6 μg/g of LPS (Figure 1), 100 g of brown rice per meal, containing an equivalent amount of LPS, is sufficient to prevent lifestyle-related diseases. Other than LPS, brown rice contains high amounts of functional components such as γ-oryzanol (21), which is an anti-oxidative polyphenol, and vitamin B1, thus rendering it an excellent functional food (22). Taken together, dewaxed brown rice can be considered as a functional agricultural product that has the potential to prevent various diseases. In the future, we will aim to demonstrate the preventative and therapeutic effects of dewaxed brown rice on modern diseases by conducting human intervention studies and/or studies using animal disease models.

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Conflicts of Interest

The Authors have no financial conflicts of interest.

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