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An Extract From Brown Rice Inhibits Signal Transduction of Angiotensin II in Vascular Smooth Muscle Cells

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BACKGROUND
Health benefits of brown rice over white rice have been described previously. However, whether the outer bran of rice contains an ingredient useful to prevent cardiovascular diseases remains unknown. The subaleurone layer of rice, which is usually lost by milling brown rice for whitening, is rich in varied nutrients, suggesting that some ingredient contained within this layer may be beneficial for the cardiovascular system.

METHODS
To assess potential benefits of the subaleurone layer toward pathological vascular remodeling, we examined the effects of the layer extracts from Japanese rice (*Oryza sativa* var. japonica) on angiotensin II (Ang II)-induced signal transduction and hypertrophy in cultured rat vascular smooth muscle cells (VSMCs).

RESULTS
Pretreatment of the ethyl acetate extract (100 μg/ml), but not other extracts, inhibited Ang II (100 nmol/l)-induced immediate signal transduction events. Also, the extract diminished c-Fos expression and hypertrophic protein accumulation induced by Ang II in the cells.

CONCLUSION
These data suggest that an ingredient in the ethyl acetate extract from the subaleurone layer of rice has a protective effect toward cardiovascular diseases by interfering with signal transduction induced by Ang II.

Keywords: *angiotensin II; blood pressure; hypertension; rice; signal transduction; vascular smooth muscle cell*

American Journal of Hypertension, advance online publication 17 February 2011; doi:10.1038/ajh.2011.10

White rice has long been consumed as a staple food in many countries. However, it has been reported that white rice consumption may increase the risk for type II diabetes.[1] In contrast, brown rice intake seems to decrease risks of cardiovascular disease and diabetes,[2] suggesting that the potential health benefit of rice is lost during rice milling. The subaleurone layer of rice located between the white center and outer bran is usually lost in the milling process. This layer contains various important nutrients, and recent developments in pressure-equalizing rice polishing technology have enabled producers to retain this layer on certain types of white rice (http://www.toyo-rice.jp/english/index.html). However, despite proposed health benefits, knowledge about whether this rice layer contains an ingredient that prevents cardiovascular diseases remains unknown.

Angiotensin II (Ang II), the major bioactive peptide of the renin–angiotensin system, is strongly implicated in various cardiovascular diseases such as hypertension, atherosclerosis, and heart failure.[3] In addition to inducing arterial contraction, Ang II is an important growth factor causing hypertrophy and or hyperplasia of vascular smooth muscle cells (VSMCs). Most of the known actions of Ang II are mediated through the angiotensin type 1 receptor (AT_1), which is coupled to multiple heterotrimeric G proteins.[4] We and others have previously reported that Ang II induces hypertrophy and migration of VSMCs through the epidermal growth factor receptor (EGFR) and Rho-kinase (ROCK), which are mainly activated through G_q and $G_{12/13}$, respectively.[5–7] The activation of the EGFR and ROCK by Ang II can be monitored by phosphorylation of the receptor and the substrate myosin phosphatase target subunit-1 (MYPT1) in VSMCs, respectively.[6] To assess a cardiovascular protective effect of the rice subaleurone layer, we have investigated the effects of its extracts on Ang II–induced signal transduction events and subsequent hypertrophy and migration in cultured VSMCs.

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Received 27 October 2010; first decision 10 November 2010; accepted 29 December 2010.

METHODS

Reagents. The subaleurone layer of Japanese rice (*Oryza sativa* var. japonica) was separated with pressure-equalizing rice polishing technology developed by Toyo Rice, Tokyo, Japan (http://www.toyo-rice.jp/english/index.html). Ang II was purchased from Sigma-Aldrich (St Louis, MO). Antibodies for Tyr^{1068}-phosphorylayed EGFR and Thr^{853}-phosphorylated MYPT1 were purchased from Invitrogen (4478G: Carlsbad, CA) and Upstate (36-003; Millipore, Billerica, MA), respectively. Antibodies for EGFR and c-Fos were purchased from Santa Cruz Biotechnology (sc-03 and sc-52; Santa Cruz, CA). Antibody for MYPT1 was purchased from Covance (PRB-457C; Princeton, NJ). Antibody for GAPDH was purchased from Chemicon, Billerica, MA (MRB374; Millipore).

Extraction of the rice layer. The subaleurone layer of Japanese rice (1.2 kg) was refluxed with methanol at 60 °C to produce a methanol extract. The extract was filtered and evaporated using a rotary evaporator under reduced pressure to yield a viscous mass (140 g). This extract was suspended in water (1 liter) and transfered to a 2–l funnel for separation. The suspended solution was extracted three times with 300 ml of hexane, dichloromethane, and ethyl acetate, successively. Each soluble fraction was concentrated under reduced pressure at 40 °C to give hexane (60.0 g), dichloromethane (1.11 g), and ethyl acetate (2.19 g) extracts. Each extract was reconstituted in 100% dimethyl sulfoxide at 100 mg/ml. Extracts prepared at three distinct times were utilized to confirm the findings.

Cell culture. VSMCs were prepared from thoracic aorta of Sprague-Dawley rats by the explant method as described previously.[8] All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Temple University. VSMCs were subcultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. VSMCs from passages 3–12 used in the experiments typically showed >99% positive immunostaining of smooth muscle α-actin antibody. Cells at 80–90% confluence in culture wells were made quiescent by incubation with serum-free medium for 2–3 days. To avoid any potential phenotypic selection and genetic alteration, VSMCs were renewed every 2–3 months and VSMCs from frozen stock were never used. The results were confirmed in at least two distinct cell lines. For experiments, the serum-free media was exchanged and VSMCs were incubated with fresh serum-free media for 30 min. Cells were then pretreated with the rice layer extract (final concentration 10, 30, or 100 μg/ml in Dulbecco's modified Eagle's medium with 0.1% dimethyl sulfoxide) or the vehicle (0.1% dimethyl sulfoxide) as a control for 1h before Ang II stimulation.

Immunoblotting. Immunoblotting was performed as previously described.[8] Quiescent VSMCs grown on 6-well plates (~5×10^5 cells/well) were stimulated with Ang II for specified durations. The reaction was terminated by the replacement of medium with 100 μl of 1 × SDS sample buffer. Each 40 μl of the cell lysates was subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4 °C. After incubation with the peroxidase-linked secondary antibody for 1 h at room temperature, immunoreactive proteins were visualized by a chemiluminescence reaction kit. The results were quantified by densitometry in the linear range of film exposure using CanoScan N670U (Canon, Lake Success, NY) and Un-Scan-It Gel 5.3 software (Silk Scientific, Orem, UT), and the ratios EGFR-p/EGFR and MYPT1-p/MYPT1 were calculated. An example of data supporting the linearity has been demonstrated.[7] Unless stated otherwise, results were expressed as % increase in which the response to Ang II is defined as 100% because the basal signals are more varied depending on film exposure than the stimulated signals.

Protein assay. To assess Ang II–induced VSMC hypertrophy directly, we have been utilizing an assay measuring cell protein accumulation but not a radiolabeled leucine incorporation assay to avoid unnecessary use of a radioactive compound.[6,9] Consistent with a highly cited past report,[10] incubation of VSMCs with 100 nmol/l Ang II for 3 days in serum-free Dulbecco's modified Eagle's medium resulted in increases in cell protein and volume without any significant change in cell proliferation/viability.[6,9] Thus, the protein assay was performed in the present study to estimate protein synthesis as previously described.[6,9] VSMC on 12-well culture plates were incubated with serum-free Dulbecco's modified Eagle's medium for 2 days and were further incubated with or without 100 nmol/l Ang II for 3 days. After aspiration of the medium, cells were washed twice in ice-cold Hank's balanced salt solution, and the total amount of cellular protein was measured using a BCA protein assay kit (Pierce, Rockford, IL).

Measurement of intracellular Ca^{2+}. Intracellular Ca^{2+} was measured as described previously by using fura 2 as an indicator.[6] VSMCs (~10^5 cells/well) subcultured on coverslips were loaded with 3 μmol/l fura 2-AM at room temperature for 45 min. The fura 2 fluorescence was acquired at a frequency of 1 Hz and the intracellular Ca^{2+} values were then obtained as described.[6]

Statistical analysis. Data are presented as means ± s.e.m. Significance between groups was evaluated using one-way analysis of variance followed by Student's *t*-test with Stat View software (SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

RESULTS

We investigated which extracts (each 100 μg/ml for 1 h treatment) from the subaleurone layer affect Ang II–induced EGFR transactivation as assessed by its Tyr^{1068} phosphorylation in VSMCs. The ethyl acetate extract, but not other extracts (hexane extract, dichloromethane extract, or methanol extract), has an inhibitory effect on the phosphorylation (data not shown).

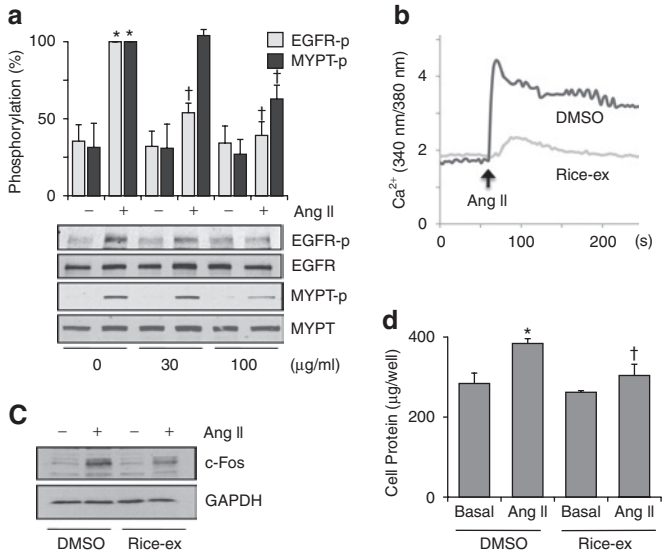

Figure 1 | An ethyl acetate extract of the subaleurone layer of rice inhibited angiotensin II (Ang II)-induced signal transduction in vascular smooth muscle cells. The cells pretreated with the extract (Rice-ex) (**a**–**d**) 100 μg/ml or (**a**) 30 μg/ml for 1 h were stimulated with 100 nmol/l Ang II for (**a**) 2 min, (**c**) 1 h, (**d**) 3 days or (**b**) indicated durations. Stimulations of epidermal growth factor receptor (EGFR) or myosin phosphatase target subunit-1 (MYPT1) (**a**) phosphorylation, (**b**) intracellular Ca^{2+} mobilization, (**c**) c-Fos induction, and (**d**) protein synthesis were evaluated as described in the Methods section. (**a**). Data are mean ± s.e.m. of three experiments. *$P < 0.05$ compared to the basal control. †$P < 0.05$ compared with the stimulated control. (**b**) Representative data from each $n = 6$. (**c**) Representative blot from $n = 3$. (**d**) Data are mean ± s.e. ($n = 3$). *$P < 0.05$ compared to the basal control. †$P < 0.05$ compared with the stimulated control. DMSO, dimethyl sulfoxide.

Pretreatment of cultured VSMCs with the ethyl acetate extract for 1 h at 30 μg/ml partially and 100 μg/ml almost completely inhibited Ang II–induced phosphorylation of EGFR, whereas only 100 μg/ml partially inhibited Ang II–induced phosphorylation of MYPT1. The extract had no effects on expressions of EGFR and MYPT1 (**Figure 1a**). No significant inhibition was observed with 10 μg/ml of the extract. Preliminary experiments with the ethyl acetate extract (100 μg/ml) for 30-min treatment showed weaker inhibitory effect on Ang II–induced EGFR phosphorylation.

We have shown that intracellular Ca^{2+} elevation through the G_q-coupled AT_1 receptor is essential for Ang II–induced EGFR transactivation.[6,8] As shown in **Figure 1b**, the acetate extracts almost completely inhibited intracellular Ca^{2+} elevation induced by Ang II. Because c-Fos induction is implicated in the Ang II–induced vascular remodeling,[11] we further examined the effects of the acetate extract on the c-Fos induction. As shown in **Figure 1c**, pretreatment of the acetate extract partially inhibited Ang II–induced c-Fos expression. Finally, we investigated whether the acetate extract inhibits Ang II–induced cell hypertrophy by measuring protein accumulation in response to Ang II. Again, the extract significantly inhibited Ang II–induced protein accumulation (**Figure 1d**). No morphological difference or reduction of total protein was observed in VSMCs treated with or without the extract for 72 h, thus excluding a toxicity of the extract.

DISCUSSION

VSMC hypertrophy induced by Ang II is believed to be pivotal for progression of hypertension.[3,5,12] Thus, inhibition of hypertrophic signal transduction events represents a potentially important therapeutic strategy for prevention of hypertensive multiorgan damage. Although our data were collected in cultured vascular cells with limited concentrations and time points, we have shown that the ethyl acetate extract from subaleurone layer of Japanese rice significantly inhibited key signaling events that have been linked to vascular remodeling *in vivo*.[5]

A number of past publications have reported that certain extracts or purified chemicals from plants have significant inhibitory effects on signal transduction events and subsequent downstream functions induced by Ang II in VSMCs.[13–15] Those plant-derived biochemicals act on either Ang II binding to the receptor[16] or the downstream signal transduction.[13–15] However, to the best of our knowledge, no past publication is available reporting if any extract from a staple grain has an inhibitory effect toward Ang II or reporting the chemical property of the rice subaleurone layer.

Our past research has pointed out the critical role of G_q-mediated intracellular Ca^{2+} elevation for downstream signal transduction of Ang II, including EGFR transactivation.[6,8] However, $G_{12/13}$ activation mainly mediates MYPT1 phosphorylation by Ang II through ROCK, and G_q makes only a partial contribution to the phosphorylation in VSMCs.[7] Therefore, it is likely that the ethyl acetate extract interferes with the signaling events between the receptor binding and the Ca^{2+} elevation such as G_q or phospholipase C. This also explains the partial inhibition of c-Fos induction by the extract because both the EGFR cascade and the ROCK cascade are implicated in the induction.[11,17] Accordingly, further studies are necessary to confirm that the rice subaleurone layer contains a specific ingredient useful toward preventing cardiovascular diseases and to determine its detailed mechanism in signal interference.

Acknowledgments: This work was supported by National Institute of Health grant, HL076770 (S.E.), by an American Heart Association Established Investigator Award, 0740042N (S.E.), and by a Grant-in-Aid for Science, Sports, and Culture, Japan (H.U.).

Disclosure: The authors declared no conflict of interest.

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