Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation

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Tabata Y, Takano K, Ito T, Iinuma M, Yoshimoto T, Miura H, Kitao Y, Ogawa S, Hori O. Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation. Am J Physiol Cell Physiol 293: C411–C418, 2007. First published May 2, 2007; doi:10.1152/ajpcell.00095.2007.—Enhanced endoplasmic reticulum (ER) stress has been implicated in various pathological situations including inflammation. During a search for compounds that regulate ER stress, we identified vaticanol B, a tetramer of resveratrol, as an agent that protects against ER stress-induced cell death. Vaticanol B suppressed the induction of unfolded protein response-targeted genes such as glucose-regulated protein 78 (GRP78) and C/EBP-homologous protein (CHOP) after cells were treated with ER stressors. Analysis in the mouse macrophage cell line RAW 264.7 revealed that vaticanol B also possesses a strong anti-inflammatory activity. Production of a variety of inflammatory modulators such as tumor necrosis factor-α, nitric oxide, and prostaglandin E2 was inhibited by vaticanol B to a much greater extent than by monomeric or dimeric resveratrol after exposure of cells to lipopolysaccharide. Further investigations to determine the common mechanisms underlying the regulation of ER stress and inflammation by vaticanol B disclosed an important role for vaticanol B in regulation of basic gene expression and in prevention of the protein leakage from the ER into the cytosol in both conditions. These results suggest that vaticanol B is a novel anti-inflammatory agent that improves the ER environment by reducing the protein load on the ER and by maintaining the membrane integrity of the ER.

SECRETORY PROTEINS UNDERGO co- and posttranslational modifications including N-linked glycosylation, disulfide bonding, and oligomeric assembly in the endoplasmic reticulum (ER). Exposure of cells to conditions such as glucose starvation, inhibition of protein glycosylation, disturbance of Ca2+ homeostasis, and oxygen deprivation accumulates unfolded proteins in the ER (ER stress). Eukaryotic cells respond to ER stress by activating a set of pathways known as the unfolded protein response (UPR) (23). The UPR is transmitted through the activation of ER resident proteins, such as Ire1α/β, protein kinase-like ER kinase (PERK), and activating transcription factor (ATF)6, and the UPR-targeted genes include molecular chaperones, folding catalysts, subunits of translocation machinery (Sec61 complex) in the ER, ER-associated protein degradation (ERAD) molecules, and antioxidant genes (9, 23, 28). However, when the protein load in the ER exceeds its folding capacity, or dome defects in the ER stress response exist, cells tend to die, typically with apoptotic features: ER stress-induced cell death (23). Important roles for ER stress and ER stress-induced cell death have been reported in a broad spectrum of pathological situations, including inflammation and infection. ER stress, caused by the accumulation of class I major histocompatibility complex proteins in the ER, plays a crucial role in skeletal muscle damage in autoimmune myositis (18). Release of arachidonic acid from the ER membrane following the activation of cytosolic phospholipase A2 (cPLA2) also induces ER stress by perturbing the integrity of the ER membrane, and it participates in the progression of C5b-9-mediated glomerular epithelial cell injury (2). Hepatitis C virus subgenomic replicons not only induce ER stress but also alter the UPR (26). In many cases, the UPR protects cells. The deletion or reduced expression of the PERK gene renders cells hypersensitive to cytokine- or complement-induced inflammation (2, 17). However, in some cases, an enhanced ER stress response contributes to the progression of inflammation through the activation of a key transcriptional factor, NF-κB (5, 10, 12, 18, 19), or an ER membrane-anchored transcriptional factor, CREBH (29).

Homocysteine-induced ER protein (Herp) is a membrane-bound, ubiquitin-like protein that is located in the ER (8, 14). Although Herp is strongly induced by ER stress or deep hypoxia, it decays rapidly as a consequence of proteasome-mediated degradation, indicating the possible contribution of Herp to the ERAD. We recently reported (8) that targeting disruption of the Herp gene rendered F9 embryonic carcinoma cells vulnerable to ER stress. The ER stress-induced death in F9 Herp-null cells was associated with aberrant ER stress signaling, structural changes in the ER, and caspase activation. Using this cell line, we evaluated the protective effect of vaticanol B, a tetramer of resveratrol that was isolated from the stem bark of Vatica rassak (25), protects cells against ER stress-induced cell death. We report here that vaticanol B suppressed the induction of UPR-targeted genes such as glucose-regulated protein 78 (GRP78) and C/EBP-homologous protein (CHOP) after cells were treated with ER stressors including tunicamycin (Tm), an inhibitor of N-linked glycosylation, and thapsigargin (Tg), an inhibitor of Ca2+ reuptake into the

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ER through sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs). Analysis in the mouse macrophage cell line RAW 264.7 also revealed that vaticanol B possesses a strong anti-inflammatory activity when cells are exposed to lipopolysaccharide (LPS). Further investigations disclosed an important role for vaticanol B in both regulation of basic gene expression and maintenance of the membrane integrity of the ER. These results suggest that vaticanol B is a novel anti-inflammatory agent that improves the ER environment, and it could be a therapeutic target for both inflammatory diseases and ER stress-related diseases.

**EXPERIMENTAL PROCEDURES**

Cell cultures and stress conditions. F9 Herp-null cells were developed as described previously (8) and maintained in DMEM containing 20% FBS. Mouse macrophage RAW 264.7 cells were provided by Dr. Naofumi Mukaida (Kanazawa University) and maintained in RPMI 1640 including 10% FBS. ER stress was induced by treating cells with Tm (0.8–2 μg/ml; Sigma, St. Louis, MO), Tg (0.3 μM; Sigma) or A-23187 (1 μM; Sigma) for the indicated times (6–48 h). To measure cell viability, cells were treated with compounds in the presence or absence of ER stressors. To estimate the induction of the UPR-targeted genes, the cells were pretreated with each compound for the indicated times (16 h), followed by treatment with stressors in the presence of the same compound for 6 h. The inflammatory response was estimated after RAW 264.7 cells were treated with 0.1 μg/ml LPS (Escherichia coli 0128:B12; Sigma) for the indicated times.

Compounds and cell viability assays. One hundred and three plant-derived compounds (IN1–IN103), including stilbenoids, xanthonoids, and flavonoids, were examined for their protective effects against ER stress in F9 Herp-null cells as described previously (24). Resveratrol (3,4',5-trihydroxy-trans-stilbene; IN4), e-viniferin (a dimer of resveratrol; IN5) and vaticanol B (a tetramer of resveratrol; IN40) were prepared by the usual methylation [(CH\(_3\))\(_2\)SO\(_4\), K\(_2\)CO\(_3\) in acetone] and acetylation (anhydroacetic acid in pyridine). Dantrolene, 1640 including 10% FBS. Mouse macrophage RAW 264.7 cells were provided by Dr. Tesque, Kyoto, Japan) as described previously (8). Cell viability was measured by the 3-(4,5-dimethyl-2-thiasele-stilbene) (LPS). Further investigations disclosed an important role for vaticanol B in both regulation of basic gene expression and maintenance of the membrane integrity of the ER. These results suggest that vaticanol B is a novel anti-inflammatory agent that improves the ER environment, and it could be a therapeutic target for both inflammatory diseases and ER stress-related diseases.

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**RESULTS**

Prevention of ER stress-induced cell death by vaticanol B. We previously reported (24) that dantrolene, an antagonist of the ryanodine receptors in the ER, and some antioxidants such as α-tocopherol partly prevented Tm-induced cell death in F9 Herp-null cells. With these agents as positive controls, 103 plant-derived compounds were screened in F9 Herp-null cells treated with Tm. Vaticanol B, a resveratrol tetramer isolated from the stem bark of *V. rassak* (IN8, Fig. 1A), was identified as an agent that may protect against ER stress. When F9 Herp-null cells were treated with Tm (0.8 μg/ml), their viability dropped to 38 (SD 4%) of the control (nonstressed) cells at 48 h (Fig. 1B). Addition of vaticanol B to cells with Tm
improved their viability to 72 (SD 6)% (Fig. 1B). In contrast, monomeric resveratrol (IN4, Fig. 1A) and ε-viniferin, a dimer of resveratrol (IN5, Fig. 1A), were less effective at improving cell viability under the same conditions (Fig. 1B). The dantrolene (30 μM)-equivalent protective activity of vaticanol B was obtained at 20 μM (Fig. 1B). To assess the protective effects of vaticanol B against ER stress, F9 Herp-null cells were treated with Tm (2 μg/ml) or Tg (0.3 μM) for 6 h, and the levels of expression of both GRP78, a target gene of the ATF6 and Ire1-XBP1 pathways, and CHOP, a target gene of the PERK-eIF2α pathway, were analyzed. The induction of both genes was observed after Tm and Tg treatment, but it was suppressed by cotreatment of cells with vaticanol B for 6 h after pretreatment for 16 h (Fig. 1C, I and II). In contrast, resveratrol was not effective in the same conditions (Fig. 1CI). These results suggested that protection of the cells against ER stress by vaticanol B was not related to the activation of the UPR, which is the case for polymethoxyflavones (24), but due to improvements in the ER environment, which reduces ER stress after treating cells with ER stressors. In the nonstressed condition, vaticanol B slightly suppressed cell growth [85 (SD 5)% of control cells] after 48 h (Supplemental Fig. 1A), while it did not induce cell death (Supplemental Fig. 1B) (the online version of this article contains supplemental data).

Role of hydroxyl groups of vaticanol B in protecting against ER stress. To assess the role of the hydroxyl groups in vaticanol B in the regulation of ER stress, two derivatives of vaticanol B, methyl ether (IN39) and acetate (IN40), were investigated for their ability to protect against ER stress. When F9 Herp-null cells were treated with Tm as described above, neither IN39 nor IN40 improved cell viability compared with vaticanol B (Fig. 2B). A consistent observation was that neither IN39 nor IN40 suppressed the induction of the UPR-targeted genes in response to Tm treatment (Fig. 2). In contrast, neither IN39 nor IN40 suppressed the induction of the UPR-targeted genes in response to Tm treatment (Fig. 2).

Anti-inflammatory properties of vaticanol B. We hypothesized that vaticanol B may also possess anti-inflammatory effects, based on accumulating evidence regarding the anti-inflammatory properties of both resveratrol (22) and ε-viniferin (6). When mouse macrophage RAW 264.7 cells were stimulated with LPS (0.1 μg/ml) for 90 min, TNF-α, a primary mediator of endotoxin, was induced and secreted into the medium (Fig. 3A, I and II). Coincubation of the cells with vaticanol B, along with preincubation for 16 h, blocked both the secretion (Fig. 3AI) and the induction (Fig. 3AI) of TNF-α at a concentration range of 5–20 μM. LPS-induced accumulation of nitrite, a metabolite of NO, in the medium was almost completely abolished by vaticanol B treatment after exposure of the cells to LPS for 16 h (Fig. 3Bl). This inhibitory effect of
vaticanol B on NO production was associated with the prevention of production of iNOS in response to LPS stimulation (Fig. 3BII). Induction of COX-2, another proinflammatory enzyme that produces eicosanoids, was also inhibited by vaticanol B, but to a lesser extent. LPS-induced production of eicosanoids such as PGE2 (Fig. 3CI) and TxB2 (data not shown) was also prevented by vaticanol B and AACOCF3; the latter is an inhibitor of cPLA2 (Fig. 3Cl). The inhibitory effect of vaticanol B on eicosanoid production was associated with both prevention of cPLA2 activation (Fig. 3CII), which was determined by the phosphorylation of cPLA2, and the regulation of COX-2 expression described above (Fig. 3BII). Resveratrol or e-viniferin had much less effective anti-inflammatory properties throughout all of these experiments (Fig. 3).

Regulation of gene expression by vaticanol B. The effect of vaticanol B on general protein synthesis was analyzed in RAW 264.7 cells after LPS treatment, because inflammation induces a variety of secretory proteins that could add to the protein load on the ER. Metabolic labeling was performed with [35S]-Met/Cys (200 μCi/condition) for 90 min in the presence or absence of LPS, and cell lysates were subjected to autoradiography (Fig. 4A) or CBB staining (Fig. 4AI). Treatment of the cells with LPS enhanced de novo protein synthesis to 150 (SD 22)% in the control (nontreated) cells (Fig. 4A, I and III). Cotreatment of the cells with vaticanol B after pretreatment for 16 h reduced de novo protein synthesis to 68 (SD 9)% of the control (nontreated) cells after LPS treatment (Fig. 4A, I and III), suggesting that, besides its anti-inflammatory effects, vaticanol B may regulate basic gene expression. In fact, treatment of cells with vaticanol B in the absence of LPS reduced de novo protein synthesis to 62 (SD 8)% of control (nontreated) cells (Supplemental Fig. 2). In contrast, resveratrol had a much weaker effect on protein synthesis under the same conditions (Fig. 4A, I and II; Supplemental Fig. 2). To further assess the effect of vaticanol B on basic gene expression, the Renilla luciferase assay was performed in RAW 264.7 cells. Cells were transfected with different types of reporter constructs including the SV40 enhancer/promoter, the CMV enhancer/promoter, and the TK promoter for 5 h, and then they were treated with resveratrol, vaticanol B, or medium alone for another 16 h. Under these conditions, cell numbers were not significantly changed based on the MTT assay (Fig. 4BI). Treatment of the cells with vaticanol B (5–20 μM) strongly reduced SV40- and TK-dependent gene expressions (Fig. 4BI, I and II) but did not reduce CMV-dependent gene expression at all (Fig. 4BIII), suggesting that vaticanol B regulates gene expression in an enhancer/promoter-specific manner. In contrast, resveratrol had almost no effect at the same doses (Fig. 4B).

Effect of vaticanol B on membrane integrity in ER. Because the impairment of the membrane integrity of the ER is linked to both inflammation and ER stress, the distribution of the ER luminal proteins was analyzed to monitor membrane integrity as described previously (2, 27). When RAW 264.7 cells were treated with either LPS or ER stressors such as Tm, Tg, or the Ca2+- ionophore A-23187 for 16 h, the redistribution of the ER luminal proteins such as GRP78, GRP94, and PDI was observed from the ER to the cytosol (Fig. 5AI). CBB staining confirmed the equivalent amounts of proteins applied to the gel (Fig. 5AII). Protein redistribution was not observed for calnexin, a membrane protein in the ER, or mHSP70/GRP75, a mitochondrial matrix protein (Fig. 5AIII), suggesting specific protein leakage from the ER lumen to the cytosol in response to both inflammation and ER stress. Cotreatment of the cells with vaticanol B (IN8), but not with resveratrol (IN4), after pretreatment for 6 h prevented the redistribution of the ER luminal proteins in response to both LPS and Tm treatment (Fig. 5, BI and CI). Interestingly, AACOCF3, but not BEL, an inhibitor of cytosolic Ca2+-independent phospholipase A2,
revealed similar inhibitory effects on the redistribution of the ER luminal proteins in response to LPS and, to a lesser extent, Tm treatment (Fig. 5, B, II and IV, and C, II and IV). Cx, a potent protein synthesis inhibitor that lessens the protein load on the ER, also prevented both LPS- and Tm-induced protein redistribution (Fig. 5, BIII and CIII). These results suggest an important role for vaticanol B in the maintenance of the membrane integrity of the ER, which is likely to be associated, at least in part, with the regulation of cPLA2 activation and protein synthesis.

**DISCUSSION**

Stilbenoids are plant-derived polyphenolic compounds that exist in a variety of forms ranging from monomers to oligomers (13, 20). A monomeric stilbenoid, resveratrol, is present in higher levels in grapes and wine compared with its oligomers. Resveratrol was originally identified as a phytoalexin and is produced in response to fungal infection or UV light irradiation (15). An increasing number of investigations on cellular and animal models have demonstrated beneficial bioactivities of resveratrol and its oligomers. These include antioxidative activity (4), anticarcinogenic activity (7, 13, 16), anti-inflammatory activity (11), antiatherogenic activity (4), antifungal activity (7), and antivirus activity (3). Among resveratrol oligomers, vaticanol C, an isomer of vaticanol B, exerted the strongest cytotoxic property against a variety of cancer cell lines, mainly by reducing mitochondrial membrane potential and activating caspases (13). Interestingly, vaticanol B did not show clear cytotoxicity against the same cancer cell lines with the exception of two, SH-SY5Y and HL60 (13).

In the present study, we identified vaticanol B as a protective agent against ER stress in F9 Herp-null cells. Comparative analysis between resveratrol and its oligomers such as /H9280-viniferin and vaticanol B revealed important roles for oligomeriza-
tion and the hydroxyl groups in terms of their protective properties. However, our preliminary results revealed that vaticanol C was cytotoxic and failed to protect F9 Herp-null cells against ER stress, suggesting that the three-dimensional structure of vaticanol B may also play an important role in protecting cells against ER stress. Further analysis revealed that the protective effect of vaticanol B against ER stress was not derived from the activation of the UPR, which is the case for polymethoxyflavones (24). Instead, vaticanol B prevents the induction of two major UPR-targeted genes, GRP78 and CHOP, in response to ER stress, suggesting that vaticanol B improves the ER environment and reduces ER stress. Although some antioxidants such as α-tocopherol partly protected F9 Herp-null cells against ER stress, and resveratrol has been reported to have some antioxidative properties (4), our unpublished results show that vaticanol B does not rescue F9 Herp-null cells from oxidant stress.

Experiments in the mouse macrophage cell line RAW 264.7 show that vaticanol B also possesses a strong anti-inflammatory property. Production of a variety of inflammatory modulators such as TNF-α, NO, and PGE₂ was inhibited by vaticanol B to a much greater extent than by monomeric or dimeric resveratrol after cells were exposed to LPS. The anti-inflammatory property of vaticanol B is likely to be derived, at least in part, from the regulation of proinflammatory gene expression and the prevention of eicosanoid production. Metabolic labeling and the Renilla luciferase assay revealed that vaticanol B not only prevented the enhancement of gene expression in response to LPS challenge, but also suppressed basic gene expression in nonstimulated cells through, at least in part, the enhancer/promoter-specific mechanism. In this regard, it is intriguing that resveratrol and other polyphenols have been reported to regulate gene expression by controlling NF-κB activation and/or chromatin remodeling (21). Although further investigation is required to clarify the way in which vaticanol B regulates basic gene expression, some of the bioactivities of resveratrol oligomers such as their antifungal (7) and anti-HIV (3) activities may be associated with this property.

Besides enhancing gene expression, inflammation is likely to be more directly associated with ER stress through the disturbance of the membrane integrity of the ER, which can be monitored by the leakage of the ER luminal proteins into the cytosol (1, 2). Furthermore, ER stress itself could impair the membrane integrity of the ER by altering Ca²⁺ homeostasis and/or by affecting the structure of the ER. In the present study, the redistribution of the ER luminal proteins such as GRP78, GRP94, and PDI was observed after treatment of RAW 264.7 cells with either LPS or ER stressors. It is less likely that these results reflect the changes in the ERAD activity (retrograde translocation of proteins from the ER to the cytosol and degradation by the ubiquitin-proteasome pathway), because the redistribution of the ER proteins was restricted to the luminal proteins; calnexin, an ER membrane protein with chaperone function.

Fig. 4. Regulation of gene expression by vaticanol B. A: general protein synthesis after LPS treatment. RAW 264.7 cells (2 × 10⁵ cells/condition) were incubated with resveratrol, vaticanol B, or medium alone for 16 h and then treated with LPS (0.1 μg/ml) in the presence or absence of each compound for another 90 min. Metabolic labeling was performed by changing the medium to a Met-free medium and adding ³⁵S-Met/Cys (200 μCi/condition) at the beginning of LPS treatment. After cell lysis, protein extracts were subjected to SDS-PAGE followed by autoradiography (I) or Coomassie brilliant blue (CBB) staining (II). III: relative intensity in each lane of I was quantified as described in the text. Values shown are means (SD) of 3 experiments. B: Renilla luciferase (Rluc) assay. RAW 264.7 cells (5 × 10⁵ cells/condition) were transfected with pRL-simian virus 40 (SV40) (I), pRL-cytomegalovirus (CMV) (II), or pRL-thymidine kinase (TK) (III) for 5 h, and the medium was changed to one including resveratrol, vaticanol, or no compounds. After 16 h, cells were subjected to Renilla luciferase assay (top) or the MTT assay (bottom). Values shown are means (SD) of 4 experiments. *P < 0.005, **P < 0.001 compared with no compounds. RLU, relative light units.
**Fig. 5. Measurement of membrane integrity in the ER.**

**A**: protein leakage from the ER into the cytosol. RAW 264.7 cells (2 × 10^6 cells/condition) were treated with LPS (0.1 μg/ml), Tm (0.8 μg/ml), thapsigargin (Tg; 0.3 μM), or A-23187 (A; 1 μM) for 16 h. Cells were lysed in buffer including digitonin (40 μg/ml) and then lysed in radioimmunoprecipitation (RIPA) buffer. The protein extracts were subjected to SDS-PAGE followed by Western blotting with anti-KDEL antibody and anti-protein disulfide isomerase (PDI) antibody (I) or CBB staining (II). Similar results were obtained in 3 separate sets of experiments.

**B and C**: effects of various compounds on the protein leakage in response to LPS (B) or Tm (C) treatment. RAW 264.7 cells (2 × 10^6 cells/condition) were incubated with resveratrol or vaticanol B (BI, CI), AACOCF3 (BII, CII), cycloheximide (Cx; BIII, CIII), bromoenol lactone (BEL; BIV, CIV) or medium alone for 6 h and then treated with LPS (0.1 μg/ml; B) or Tm (0.8 μg/ml; C) in the presence or absence of each compound for another 16 h. The cells were lysed as described above and subjected to Western blotting with anti-KDEL antibody and anti-PDI antibody. Similar results were obtained in 3 separate sets of experiments.
function, did not dislocate into the cytosol. Vaticanol B, but not resveratrol, suppressed the redistribution of the ER luminal proteins in response to both inflammation and ER stress, suggesting that vaticanol B may contribute to the maintenance of the membrane integrity in both conditions. Treatment of the cells with AACOCF₃, but not BEL, also reduced protein leakage from the ER during inflammation and, to a lesser extent, during ER stress, suggesting an important role for the activation of cPLA₂ in both inflammatory and noninflammatory situations. Further examination revealed that Cx also prevented both LPS- and Tm-induced protein redistribution from the ER to the cytosol (Fig. 5, BIII and CII) at relatively lower doses (0.1–0.5 μg/ml). Our preliminary data show that higher doses of Cx (>1 μg/ml) did not prevent the protein leakage, probably because of its cytotoxicity in the conditions of long-term incubation (Hori O, unpublished observations). Together, our results suggest that the membrane integrity of the ER is regulated by a balance between the protein load in the ER and the regulation of the hydrolysis of phospholipids in the ER, and that vaticanol B improves the ER environment in relation to both aspects.

In summary, we have demonstrated that vaticanol B is a novel anti-inflammatory agent that improves the ER environment by reducing the protein load in the ER and maintaining the membrane integrity of the ER. Although further analysis is required to dissect all of the bioactivities of vaticanol B, its lower cytotoxicity could make it a candidate for use in models of both inflammatory diseases and ER stress-related diseases in vivo.

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REFERENCES