Candidate Genes and Mechanisms for 2-Methoxyestradiol–Mediated Vasoprotection

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Abstract—2-Methoxyestradiol (2-ME; estradiol metabolite) inhibits vascular smooth muscle cell (VSMC) growth and protects against atherosclerosis and vascular injury; however, the mechanisms by which 2-ME induces these actions remain obscure. To assess the impact of 2-ME on biochemical pathways regulating VSMC biology, we used high-density oligonucleotide microarrays to identify differentially expressed genes in cultured human female aortic VSMCs treated with 2-ME acutely (4 hours) or long term (30 hours). Both single gene analysis and Gene Set Enrichment Analysis revealed 2-ME–induced downregulation of genes involved in mitotic spindle assembly and function in VSMCs. Also, Gene Set Enrichment Analysis identified effects of 2-ME on genes regulating cell-cycle progression, cell migration/adhesion, vasorelaxation, inflammation, and cholesterol metabolism. Transcriptional changes were associated with changes in protein expression, including inhibition of cyclin D1, cyclin B1, cyclin-dependent kinase 6, cyclin-dependent kinase 4, tubulin polymerization, cholesterol and steroid synthesis, and upregulation of cyclooxygenase 2 and matrix metalloproteinase 1. Microarray data suggested that 2-ME may activate peroxisome proliferator-activated receptors (PPARs) in VSMCs, and 2-ME has structural similarities with rosiglitazone (PPARγ agonist). However, our finding of weak activation and lack of binding of 2-ME to PPARs suggests that 2-ME may modulate PPAR-associated genes via indirect mechanisms, potentially involving cyclooxygenase 2. Indeed, the antimitogenic effects of 2-ME at concentrations that do not inhibit tubulin polymerization were blocked by the PPAR antagonist GW9662 and the cyclooxygenase 2 inhibitor NS398. Finally, we demonstrated that 2-ME inhibited hypoxia-inducible factor 1α. Identification of candidate genes that are positively or negatively regulated by 2-ME provides important leads to investigate and better understand the mechanisms by which 2-ME induces its vasoprotective actions. **(*Hypertension*. 2010;56:964-972.)**

Key Words: 2-methoxyestradiol ■ PPAR ■ vascular smooth muscle cells ■ microarray analysis

As with cancer, abnormal cell growth plays a key role in cardiovascular diseases. For example, vascular smooth muscle cell (VSMC) proliferation is involved in vascular remodeling that occurs at sites of atherosclerosis, hypertension-induced vascular changes, and injury-induced restenosis.[1] Hence, drugs capable of inhibiting VSMC growth by targeting key mitogenic mechanisms are effective in protecting against cardiovascular disease.[2] Thus, it is not surprising that various antimitotic therapies used in cancer also protect against vascular proliferative disorders.[3]

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with no affinity for estrogen receptors that exerts anticarcinogenic effects by inhibiting growth of cancer cells and neovascularization of tumors and is in phase II clinical trials for cancer.[4,5] Consistent with the notion that anticancer drugs may be useful in cardiovascular diseases, a series of studies by us show that 2-ME is a potent inhibitor of mitogen-induced VSMC proliferation, migration, and extracellular matrix synthesis[6–8] and that 2-ME attenuates injury-induced neointima formation[9] and cholesterol-induced atherosclerosis.[10] Interestingly, sequential metabolism of estradiol to methoxyestradiols, such as 2-ME, plays a major role in mediating the inhibitory effects of estradiol on VSMC growth,[6–8] suggesting that 2-ME could also be a noncarcinogenic alternative for estrogen therapy in postmenopausal women.

Although 2-ME has therapeutic potential to treat vaso-occlusive disorders, the mechanisms by which it inhibits VSMC growth and neointima formation remain poorly defined.

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For example, whether 2-ME inhibits VSMC growth by downregulating growth-inducing pathways or by upregulating growth-inhibitory pathways remains unclear. Moreover, the receptors via which 2-ME mediates its actions remain elusive.[11]

Accordingly, the main goal of the present study was to investigate the actions of 2-ME on VSMCs. This was accomplished using 3 approaches. First, we used transcriptional profiling in VSMCs using high-density oligonucleotide microarrays to identify 2-ME–induced changes in the expression of transcripts involved in cell-cycle progression, cell motility, vasorelaxation, cholesterol homeostasis/metabolism, and plaque formation/stability that are known to influence vascular remodeling processes and VSMC growth during cardiovascular diseases (atherosclerosis, restenosis, and plaque stability) and estrogen therapy.[12,13] Second, we confirmed the main results of the microarray transcriptional outcomes by investigating the effects of 2-ME on the protein expression or activity of key molecular targets. Third, motivated by the structural similarities of 2-ME with peroxisome proliferator-activated receptor (PPAR)-γ ligands (eg, rosiglitazone) and by the fact that, similar to PPARγ ligands, 2-ME protects against metabolic syndrome-induced disorders, improves insulin sensitivity, and inhibits VSMC growth,[1,14] we investigated whether 2-ME may, in part, mediate its actions via PPARγ.

Methods

Culture of Human Aortic Smooth Muscle Cells

Female human aortic smooth muscle cells (HASMCs) in passages 6 to 8 were cultured under standard tissue culture conditions in M231 culture medium containing growth supplement (Cascade Biologics, Inc).[15] The growth medium and serum for VSMC culture were steroid/hormone free.

Microarray Assay

Subconfluent monolayers of HASMCs were treated with either vehicle (dimethyl sulfoxide; 1 μL/mL) or 3 μmol/L of 2-ME (dissolved in dimethyl sulfoxide) in the presence of 5% FCS for 4 hours (acute phase response/early gene induction) and for 30 hours (late phase). After treatment with 2-ME, the total RNA was isolated and processed for microarray analysis (for details, see the online Data Supplement at http://hyper.ahajournals.org).

Growth Studies

[^{3}H]Thymidine incorporation (index of DNA synthesis), [^{3}H]proline incorporation (index of collagen synthesis), and cell proliferation were conducted as described previously[15] (see details in the online Data Supplement Methods section at http://hyper.ahajournals.org).

Effects of 2-ME on Intracellular Mechanisms

Changes in the expression of cell-cycle regulatory proteins (cyclin B1 and cyclin D1), cyclooxygenase 2 (COX-2), and hypoxia-inducible factor 1α (HIF-1α) were analyzed by Western blots. Labeling with radioactive γ-^{32}P-adenosine triphosphate was used to assess cyclin-dependent kinase 6 (cdk6) activity. The influence of 2-ME on the dynamics of tubulin polymerization and HIF-1α was assayed by immunofluorescence microscopy and as described previously.[9]

PPAR Receptor Binding and Activation Experiments

Radioligand-based scintillation proximity assays were used to assess binding of 2-ME to PPARs, whereas PPAR luciferase reporter assay was used to measure PPAR activity (for details see the online Data Supplement Methods).

Changes in Endogenous Cholesterol, Progesterone, and Testosterone Levels

Animal handling and experimentation were in accordance with the Swiss animal protection laws. All of the protocols were approved by the institutional animal care and use committee. Male Wistar-Kyoto rats (350 to 400 g; RCC) were anesthetized using 100 mg/kg of ketamine plus 15 mg/kg of xylazine and implanted with osmotic pumps for intravenous delivery of the vehicle (n=9) or 2-ME (n=11) at a rate of 350 μg/kg per day. A 90% PEG-400 solution in saline (10%) was used as vehicle. After 14 days, the animals were euthanized and blood samples drawn and serum prepared to assess the levels of cholesterol, progesterone, and testosterone.[9]

Statistics

Microarray experiments were conducted in triplicates, whereas all of the growth experiments were performed in triplicates or quadruplicates with 3 to 4 separate cultures. Statistical analyses of microarray data were performed according to standard methods (see the online Data Supplement for details). Statistical analysis was performed using ANOVA, paired Student t test, or Fisher least significant difference test, as appropriate. A value of $P<0.05$ was considered statistically significant.

Results

We investigated both a short-term (4-hour) and a prolonged (30-hour) treatment of cultured HASMCs with 3 μmol/L of 2-ME on modulation of gene expression. Transcriptional changes after short-term treatment (4 hours) were indicative of the beginning of transcriptional responses only (see Table S1 in the online Data Supplement at http://hyper.ahajournals.org), with no statistically significant results. Hence, the changes after long-term treatment (30 hours) were considered more relevant. Indeed, after 30 hours of treatment, significant transcriptional changes were manifest. The top 20 genes that were downregulated after a 30-hour treatment with 2-ME clearly demonstrated that 2-ME interfered with key processes associated with nuclear division and cell mitosis (see Table S2). In this regard, 2-ME downregulated/inhibited genes promoting cell cycle and cell growth at multiple levels. Long-term treatment with 2-ME also upregulated several genes, 20 of which were significant (see Table S3).

Gene Set Enrichment Analysis (GSEA) identified functionally related gene sets that respond to 2-ME in a coordinated manner and are relevant for cardiovascular disease. As shown in Figure 1A, treatment with 2-ME significantly downregulated the expression of genes within the cell-cycle pathway that are essential for cell growth. Consistent with these observations, treatment of HASMCs with 2-ME downregulated the expression of cyclin D1 and cyclin B1 and inhibited cdk6 and cdk4 activity (Figure 1B). Moreover, consistent with its downregulatory effects on microtubule processes, which are critical for cell division, 2-ME inhibited tubulin polymerization, a key process for cytokinesis (Figure 1C). The cell-cycle distributions in growing cells versus serum-starved cells treated with FCS for 30 hours were similar (see Figure S1). Treatment with 2-ME did not influence HASMC viability (see Figure S2), nor did it induce apoptosis (see Figure S2) or aneuploidy (see Figure S3).

2-ME was also found to regulate genes involved in prostaglandin synthesis, as shown in Figure 2A. Importantly, Western blots of lysates from HASMCs treated with 2-ME show a

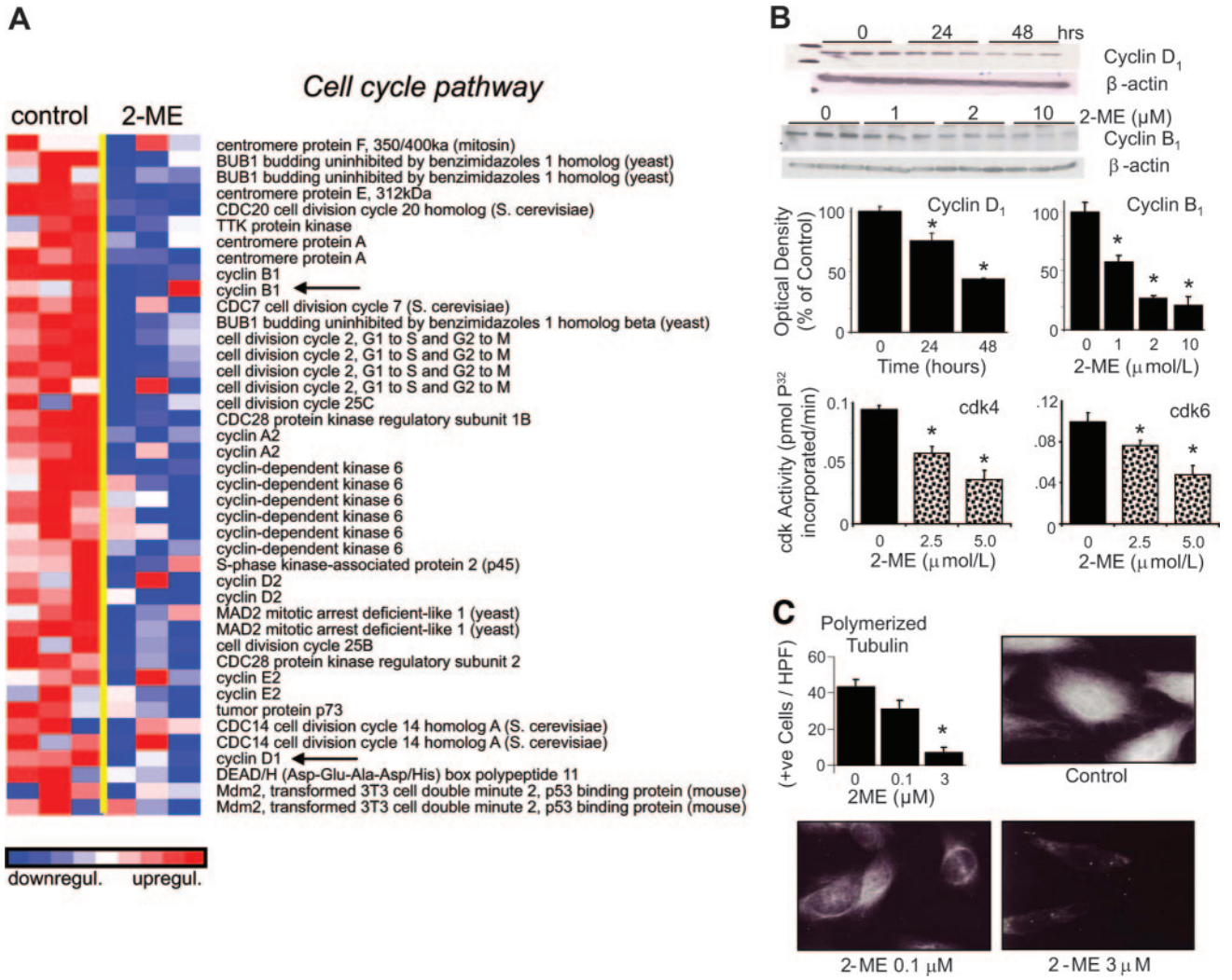

Figure 1. Inhibitory effects of 2-ME on serum-induced activation of pathways regulating cell cycle. A, The microarray analysis of transcriptional changes in HASMCs treated with 2-ME (3 μmol/L) for 30 hours. Expression levels are depicted as color scale from red (upregulation) to blue (downregulation). GSEA revealed downregulation of key genes known to induce cytokinesis. B, Western blots and bar graph (changes in optical density; mean±SEM) depicting the significant inhibitory effects of 2-ME on cyclin D1 and cyclin B1 expression and on cdk4 and cdk6 activity in HASMCs treated with 2-ME. C, Representative photomicrographs and bar graph depicting the inhibitory effects of 2-ME on tubulin polymerization in proliferating HASMCs. The tubulin polymerization experiments were conducted in triplicate. Western blotting and cdk activity data are presented as mean±SEM (n=3). *$P<0.05$ vs vehicle-treated control. Arrows indicate the genes for which protein expression was confirmed.

significant upregulation in the expression of COX-2 (Figure 2B), complementary to that observed by microarray analysis.

2-ME has been shown to lower cholesterol and protect against cholesterol-induced atherosclerosis.[10] Consistent with the cholesterol-lowering effects of 2-ME, treatment with 2-ME significantly downregulated genes involved in cholesterol synthesis (Figure 3A). Moreover, treatment of male rats with 2-ME significantly reduced the levels of total cholesterol (Figure 3B), as well as the levels of progesterone and testosterone, which require cholesterol as a precursor molecule.

As shown in Figure 4A, GSEA analysis demonstrated that 2-ME upregulated plaque-associated transcripts. Moreover, analysis of matrix metalloproteinase 1 (MMP-1) by Western blots showed that, similar to lipopolysaccharide, 2-ME induced MMP-1 expression in HASMCs (Figure 4B).

Although 2-ME is known to interact with tubulin,[5,9] the receptor via which it mediates its actions at lower concentrations remains elusive. 2-ME bears structural similarity with PPAR ligands, for example, rosiglitazone (Figure 5A), and, indeed, we found that 2-ME significantly downregulated gene transcripts known to be downregulated by rosiglitazone, as shown in Figure 5B. Moreover, within the drug resistance and metabolism pathway, 2-ME upregulated the genes for PPARα and PPARγ (see Figure S4). Hence, combining evidence from pharmacological studies and GSEA, we hypothesized that 2-ME may potentially be a PPAR ligand. PPAR receptor activation assays using luciferase reporters demonstrated weak activation of PPARγ, PPARα, and PPARδ by 2-ME (Figure 5C). Compared with known ligands, the PPAR activation by 2-ME was $\geq$10-fold weaker (see Figure S5). Moreover, radioligand binding assays showed little affinity of 2-ME for PPARs (see Table S4); moreover, inhibition of COX-2, known to generate endogenous ligands (prostaglandins/prostacyclins) for PPAR, abrogated 2-ME–

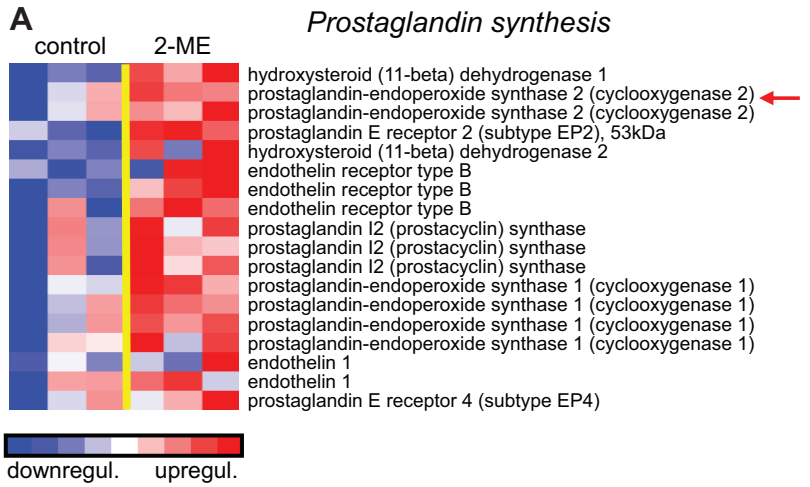

Figure 2. Stimulatory effects of 2-ME on prostaglandin synthesis in HASMCs. A, The microarray analysis of transcriptional changes in HASMCs treated with 2-ME (3 μmol/L) for 30 hours. Expression levels are depicted as color scale from red (upregulation) to blue (downregulation). GSEA revealed induction of key proteins known to facilitate prostaglandin synthesis. B, The significant stimulatory effects of 2-ME on COX-2 protein expression in HASMCs treated with 2-ME. Data are presented as mean±SEM (n=3). *$P<0.05$ vs vehicle-treated control. Arrow indicates the gene for which protein expression was confirmed.

mediated activation of PPAR (see Figure S5). Microarray results suggested that, similar to rosiglitazone, 2-ME induced transcripts related to hypoxia and the transcription factor HIF-1α. To further validate this effect, we assessed the effects of 2-ME on HIF-1α expression after hypoxia using immunofluorescence microscopy and Western blots. Exposure of HASMCs to hypoxia induced HIF-1α expression, and this stimulatory effect was abrogated in HASMCs treated with 2-ME (Figure 5D). To further investigate the possibility that 2-ME exerts effects mediated by PPAR, we tested whether the growth-inhibitory effects of 2-ME are blocked by the PPARγ antagonist GW9662.

Serum increased several-fold all indices of cellular growth including DNA synthesis, collagen synthesis, and cell proliferation. In serum-treated cells, 2-ME concentration-dependently attenuated cell proliferation (Figure 6A left), collagen synthesis (Figure 6B, left), and DNA synthesis (see Figure S6). Similar to 2-ME, rosiglitazone, a well-established PPARγ ligand, concentration-dependently inhibited FCS-induced cell proliferation (Figure 6A, right), collagen synthesis (Figure 6B, right), and

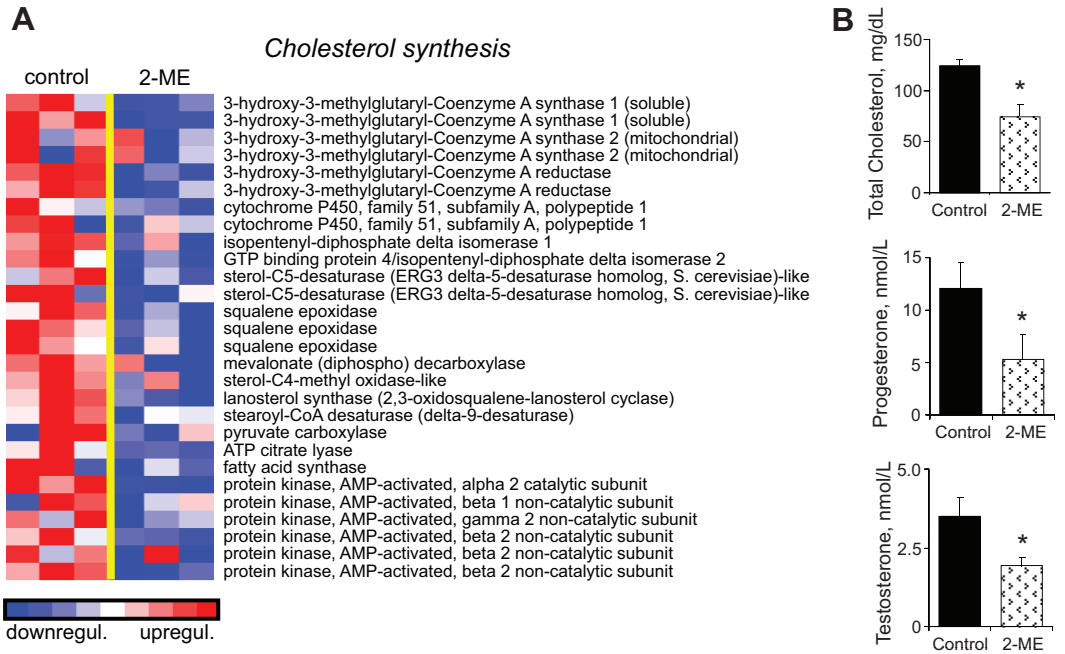

Figure 3. Inhibitory effects of 2-ME on pathways regulating cholesterol synthesis. A, The microarray analysis of transcriptional changes in HASMCs treated with 2-ME (3 μmol/L) for 30 hours. Expression levels are depicted as color scale from red (upregulation) to blue (downregulation). GSEA revealed downregulation of key genes known to facilitate cholesterol synthesis. B, The significant inhibitory effects of 2-ME on circulating levels of cholesterol, progesterone, and testosterone in male rats treated for 14 days with 2-ME. Data for cholesterol levels represent mean±SEM (n=7 placebo; n=7 2-ME treated). *$P<0.05$ vs vehicle-treated control.

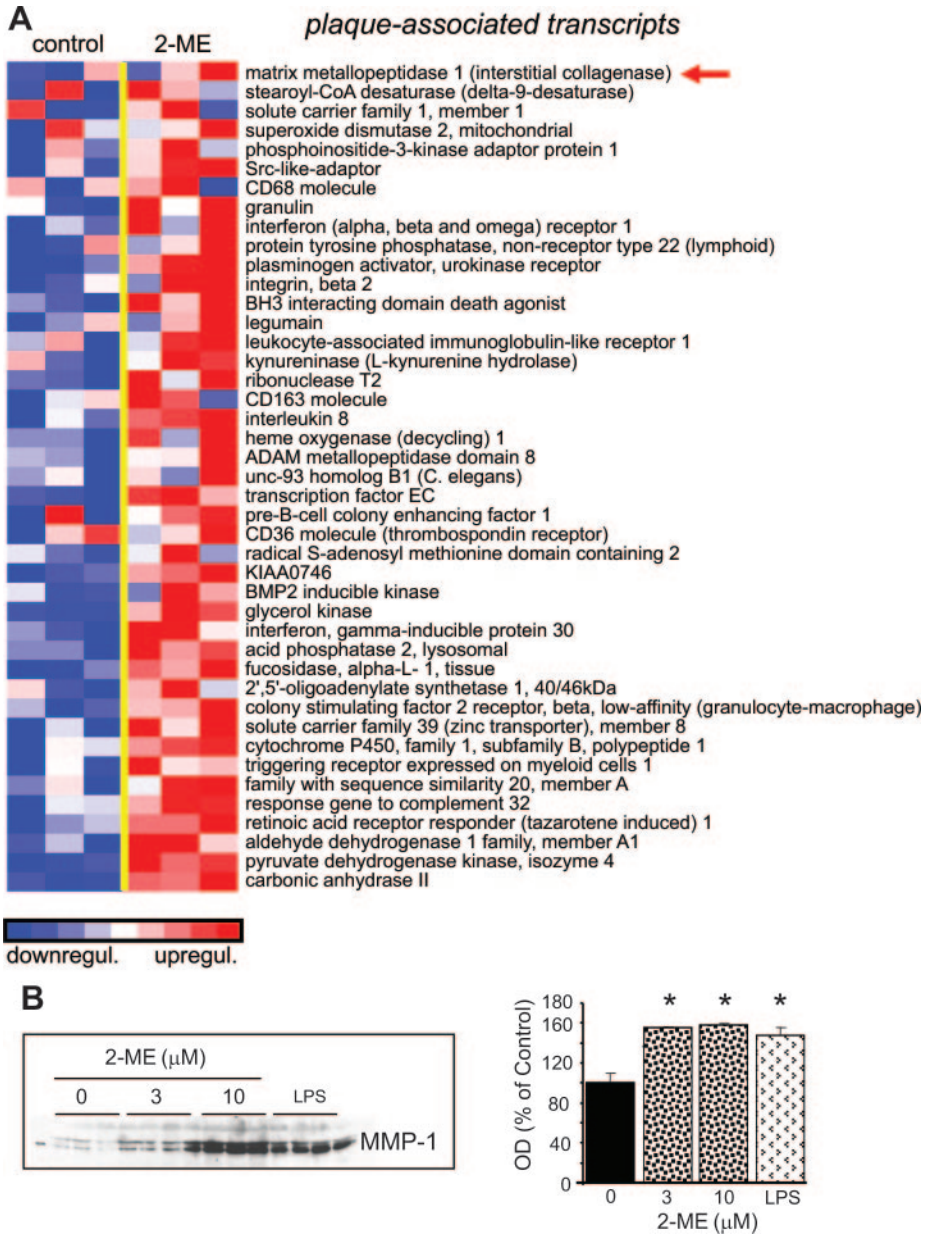

Figure 4. Stimulatory effects of 2-ME on plaque-associated regulatory molecules in HASMCs. A, The transcriptional changes in HASMCs treated with 2-ME (3 μmol/L) for 30 hours. Expression levels are depicted as color scale from red (upregulation) to blue (downregulation). GSEA revealed upregulation of key plaque-associated transcripts. B, The significant stimulatory effects of 2-ME on MMP-1 expression in HASMCs treated with 2-ME. HASMCs treated with lipopolysaccharides served as a positive control. Data for microarray analysis and Western blotting represent mean±SEM (n=3). *$P<0.05$ vs vehicle-treated control. Arrow indicates the gene for which protein expression was confirmed.

DNA synthesis (see Figure S6). As compared with rosiglitazone, 2-ME was more potent in inhibiting serum-induced HASMC growth. The lowest concentrations of 2-ME and rosiglitazone that significantly inhibited HASMC growth (DNA synthesis, cell proliferation, and collagen synthesis) were 1 nmol/L and 10 μmol/L, respectively. With 2-ME, a 50% inhibition of DNA synthesis, cell proliferation, and collagen synthesis was observed at 10, 100, and 100 nmol/L, respectively, whereas, ≥10 μmol/L of rosiglitazone was required to inhibit HASMC growth by 50%. Cotreatment with the PPARγ antagonist GW9662 blocked the inhibitory effects of rosiglitazone. Moreover, GW9662 abrogated the inhibitory effects of low (1 to 10 nmol/L) but not high (>10 nmol/L) concentrations of 2-ME on cell number (Figure 6A, left, and insert bar graph), collagen production (Figure 6B, left, and insert bar graph), and DNA synthesis (see Figure S6). Similar to GW9662, NS398 (10 μmol/L; a COX-2 inhibitor) abrogated the inhibitory effects of low (1 to 10 nmol/L) but not high (>10 nmol/L) concentrations of 2-ME on cell proliferation and DNA synthesis (Figure 6C).

Discussion

2-ME is an investigational drug in phase II clinical trials for cancer and has promise as a drug to inhibit neointima formation by inhibiting VSMC growth.[4,5,9,11] Here, using

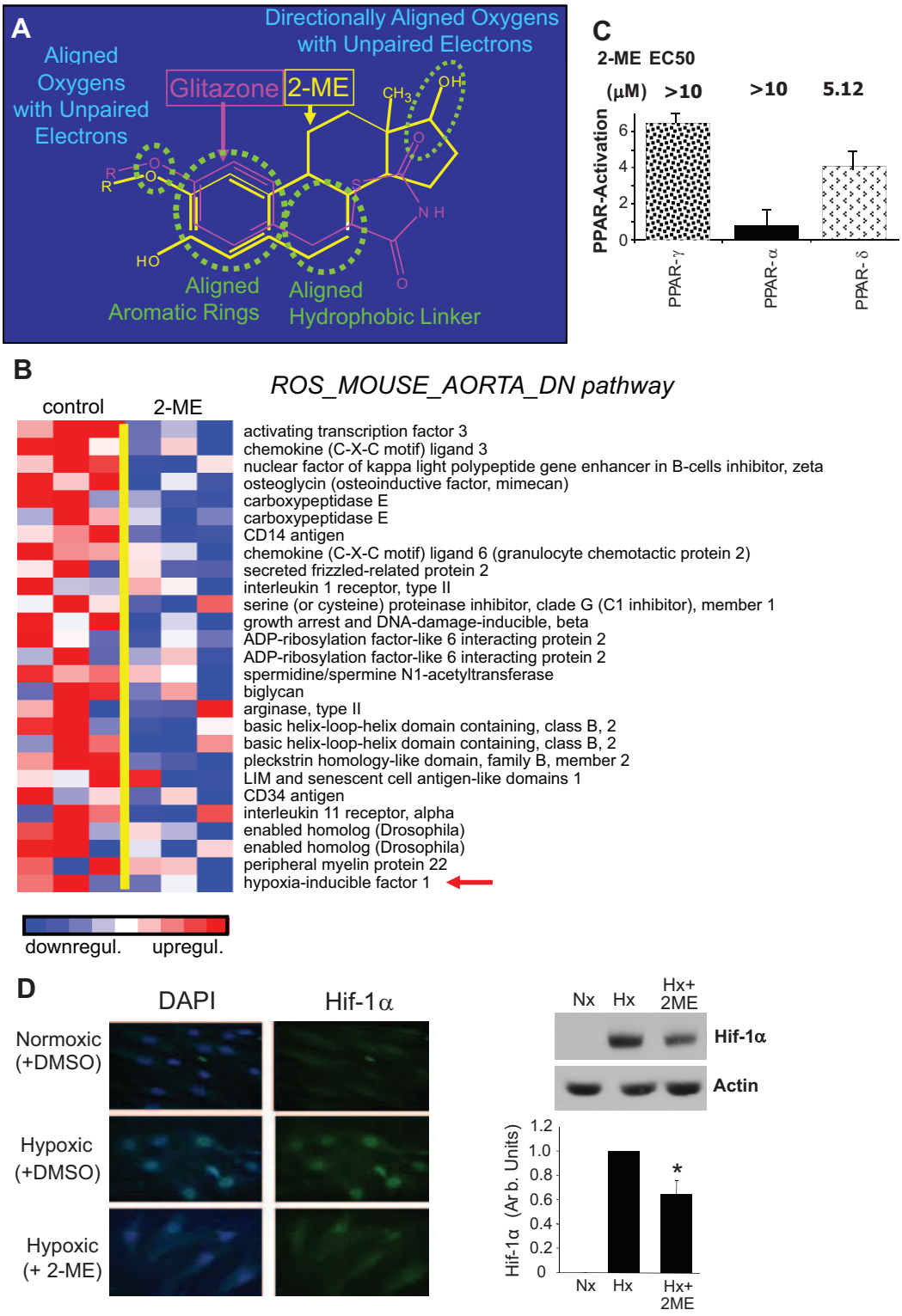

Figure 5. Inhibitory effects of 2-ME on pathways downregulated by rosiglitazone in HASMCs. A, The structural similarity between 2-ME and PPARγ ligand rosiglitazone. B, The microarray analysis of transcriptional changes in HASMCs treated with 2-ME (3 μmol/L) for 30 hours. Expression levels are depicted as color scale from red (upregulation) to blue (downregulation). GSEA revealed downregulation of key genes known to be downregulated by rosiglitazone. C, The stimulatory effects of 2-ME on PPARδ, PPARγ, and PPARα using a transactivation assay. D, Representative photomicrographs and Western blots demonstrating the inhibitory effects of 1 μmol/L of 2-ME on hypoxia (Hx) induced HIF-1α expression in HASMCs. The bar graph represents the densitometric analysis of Western blots (mean±SEM) from 3 separate experiments. Data for microarray analysis and Western blotting represent mean±SEM (n=3). The immunostaining experiments were conducted in quadruplicate. *$P<0.05$ vs HASMCs treated with hypoxia (Hx). Nx indicates normoxia. Arrow indicates the gene for which protein expression was confirmed.

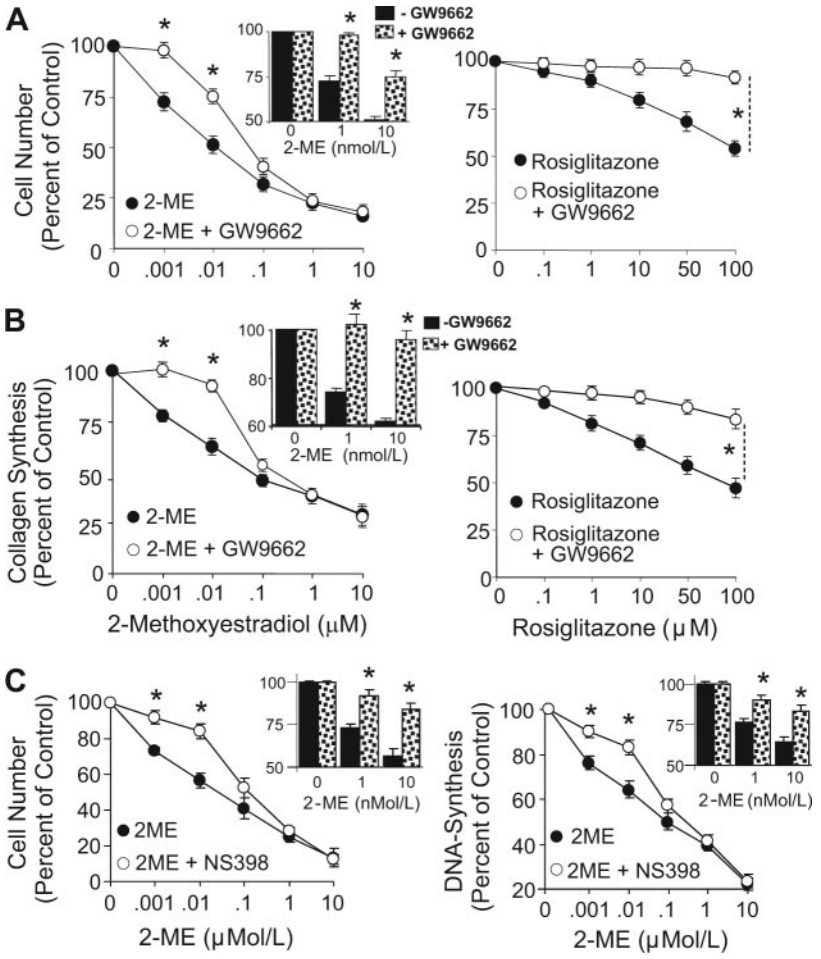

Figure 6. Depicts the attenuation by PPARγ antagonist GW9662 of the concentration-dependent inhibitory effects of 2-ME (0.001 to 10 μmol/L; left) or rosiglitazone (0.1 to 100 μmol/L; right) on 2.5% FCS-induced cell number (A) and collagen ([^{3}H]proline incorporation; B) in HASMCs. Also shown are the effects of NS398 on 2-ME–induced inhibition of cell number and DNA synthesis (C). Data represent mean±SEM from 3 separate experiments conducted in triplicates. *$P<0.05$ significant reversal of the inhibitory effects of 2-ME or rosiglitazone by GW9662 or NS398.

high-density oligonucleotide microarrays, we provide evidence that 2-ME's antivasoocclusive actions are mediated in part by regulation of specific genes. In this regard, the microarray data provide evidence that 2-ME downregulates genes that are critically involved in mitotic spindle assembly, cell-cycle progression, and cytokinesis and show that 2-ME increases the expression of genes involved in vasorelaxation (eg, COX-2/prostaglandins) and inhibits genes involved in cholesterol biosynthesis. 2-ME also causes major changes in genes regulating the respiratory chain and redox pathways, as well as plaque stability. Finally, leads from the microarray data coupled with biochemical assays provide evidence that 2-ME mimics the effects of rosiglitazone on HASMC growth, and these effects may in part be PPARγ mediated. Moreover, 2-ME significantly downregulates gene transcripts and proteins known to be similarly affected by rosiglitazone. These data support the concept that 2-ME may be an endogenous ligand for PPARs or activate PPAR-associated genes downstream from the receptor or via stimulation of an endogenous ligand, such as COX-2–derived prostaglandins. Identification of candidate genes that are positively or negatively regulated by 2-ME provides important leads to investigate the mechanisms by which 2-ME induces its vasoprotective and antivasoocclusive actions.

Abnormal growth of VSMCs plays a key role in the etiology of neointima thickening, which contributes to vasoocclusive disorders and cardiovascular disease.[1,2] Because 2-ME inhibits injury-induced neointima formation, as well as cholesterol-induced atherosclerosis,[9,10] screening of genes that are influenced by 2-ME would provide important leads to further investigate the potential role of various pathways in mediating the antiproliferative and antivasoocclusive actions of 2-ME. Our previous studies show that, as with VSMCs, 2-ME also inhibits growth of glomerular mesangial cells and cardiac fibroblasts.[16,17] It is likely that our results in HASMCs may provide important leads to the mechanisms via which 2-ME is protective against renal disease associated with glomerulosclerosis, as well as against cardiac hypertrophy.[11,18]

Although multiple mechanisms are known to regulate VSMC growth, cell-cycle regulatory proteins and tubulin play a major role in promoting VSMC proliferation, migration, and cytokinesis.[2] Screening of the genes downregulated by 2-ME provides strong evidence that 2-ME is a potent inhibitor of factors involved in cell-cycle progression. Moreover, 2-ME downregulates genes involved in tubulin polymerization, an essential step in nuclear division/cytokinesis, as well as cell migration. These conclusions are further supported by the fact that the effects of 2-ME on the expression of genes regulating the cell cycle are also reflected at the protein level for some selected proteins tested. In this context, treatment of VSMCs both downregulated genes and reduced the activity of proteins involved in the G_1 to G_2

transition. Moreover, mimicking the effects of tubulin-associated genes, 2-ME inhibited tubulin polymerization in VSMCs. Finally, the fact that 2-ME downregulates ki67 gene expression is also supported by our previous finding that 2-ME inhibits neointima formation,[9] and this effect is accompanied with inhibition of ki67-positive VSMCs.[9]

Previous studies from our group provide evidence that, in animals with the metabolic syndrome, 2-ME protects against cardiorenal disease progression and improves insulin sensitivity.[14] Our finding that, in HASMCs, 2-ME mimics the effects of rosiglitazone with respect to downregulating the expression of multiple transcripts suggests that the protective effects of 2-ME may, in part, be mediated via a PPARγ-linked mechanism. This contention is also supported by the observations that 2-ME has structural similarity to rosiglitazone and that the antimitogenic effects of 2-ME, at concentrations that do not interfere with tubulin polymerization, are blocked by the PPARγ antagonist GW9662. Our finding that 2-ME inhibits hypoxia-induced expression of HIF-1α, a transcription factor known to induce VSMC growth,[19] suggests that 2-ME may induce its vasoprotective actions by inhibiting HIF-1α. Recent findings that PPAR-γ activation inhibits HIF-1α expression in allergic airway disease in mice[20] suggest that the inhibitory effects of 2-ME on HIF-1α in VSMCs may be PPAR-γ mediated. However, it is possible that the effects of 2-ME on PPAR-γ activation and HIF-1α inhibition are independent actions of 2-ME, which synergistically inhibit VSMC growth; additional studies are required to evaluate this possibility.

Although the above observations suggest that 2-ME may mediate its effects via PPARs, radioligand-displacement studies detect no direct binding of 2-ME to PPARα, PPARγ, or PPARδ at concentrations $\leq$10 μmol/L. However, in transcriptional transactivation studies, 2-ME activates PPARγ but with lower potency than that at which it mediates its antiproliferative effects on VSMC proliferation that are reversible by a PPARγ antagonist. These data suggest that 2-ME may mediate its effects via modulation of PPARγ-dependent gene regulation but that this occurs by downstream/upstream or other regulatory events, which are independent of ligand binding. In this context, it is interesting to note that in microarray assays treatment of VSMCs with 2-ME upregulated the genes for PPAR receptors (see Figure S4), suggesting that 2-ME's effects may in part be mediated via modulation of PPAR expression. However, the facts that 2-ME induces the expression of COX-2, that COX-2 generates endogenous ligands for PPAR (eg, prostaglandin-D2/15D-prostaglandin-J2), and that inhibition of COX-2 with NS398 blocks 2-ME–mediated activation of PPAR and inhibition of VSMC growth suggest that 2-ME may indirectly influence PPAR-associated genes via generation of these endogenous ligands or via targets downstream from PPARs. Because GW9662 has been shown to inhibit COX-2 activity in VSMCs, it is possible that it abrogates the antimitogenic effects of 2-ME by inhibiting COX-2 activity,[21] similar to NS398. Indeed, the stimulatory effects of 2-ME on COX-2 were attenuated by GW9662 (see Figure S7). However, more in-depth studies are needed to investigate this aspect.

The microarray data provide evidence that, in HASMCs, 2-ME downregulates genes involved in cholesterol synthesis, suggesting that it may protect against atherosclerosis. Indeed, recent animal studies provide evidence that treatment with 2-ME protects against cholesterol-induced atherosclerosis in apolipoprotein E knockout mice[10]; moreover, 2-ME lowers cholesterol levels in normal and Zucker obese rats with the metabolic syndrome.[14] Because the mechanisms via which 2-ME lowers cholesterol levels remain unclear, the data from microarray analysis of cholesterol synthesis genes regulated by 2-ME provide invaluable leads to further investigate the mechanisms involved. Indeed, our data provide evidence that 2-ME downregulates the expression of multiple genes that play an important role in cholesterol biosynthesis. In this context, 2-ME inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), a target for statins to lower cholesterol[22]; inhibits squalene epoxidase, which acts downstream from HMG-CoA to sequentially convert squalene to 2,3-oxidosqualene and cholesterol[23]; and inhibits mevalonate (diphospho) decarboxylase, which participates in cholesterol synthesis downstream from acetyl-CoA by converting mevalonic acid to isopentenyl-PP and squalene.[24] Because cholesterol is a precursor for steroid synthesis, we investigated the levels of progesterone and testosterone in male rats treated with 2-ME. Consistent with our gene array data, along with total cholesterol, 2-ME decreased the circulating levels of both testosterone and progesterone. Because CYP450 is responsible for the conversion of cholesterol to steroids, it is feasible that inhibitory effects of 2-ME also contribute to its inhibitory effects on testosterone and progesterone levels. The effects on estradiol levels could not be assessed, because the levels in male animals were below detection limit. Taken together, our findings provide evidence for the potential gene targets via which 2-ME can reduce cholesterol synthesis and, subsequently, atherosclerosis and cardiovascular disease. This contention is also supported by the recent findings that 2-ME reduces cholesterol and inhibits atherosclerosis in female mice.[10] Because the gene expression studies were conducted in human female cells, whereas the changes in cholesterol and steroid levels were measured in male rats treated with 2-ME, additional studies in females are required to further confirm these findings.

Consistent with our microarray data, recent gene expression studies on the effects of 2-ME on tumor/cancer cells provide evidence that, similar to VSMCs, 2-ME downregulates cell cycle and tubulin-associated genes involved in the mitotic spindle assembly checkpoint and inhibits mitogenesis and migration/metastasis.[24,25] Interestingly, unlike VSMCs, 2-ME induces apoptosis via upregulation of apoptosis-associated genes in cancer cells.[26]

The beneficial effects of 2-ME on the cardiovascular system, the fact that 2-ME is nonfeminizing, and the fact that 2-ME is anticarcinogenic render 2-ME an attractive therapeutic alternative for estrogen therapy. However, because estrogen therapy induces deleterious effects in selective groups of women with pre-established plaques,[4] it is important to assess whether 2-ME may be a safer substitute to treat this group of postmenopausal women. The present study shows that 2-ME upregulates several plaque-associated transcripts. These ef-

fects are also confirmed by Western blots, where 2-ME upregulates the expression of MMP-1, which is implicated in plaque destabilization and rupture.[27] Taken together, these findings suggest that the therapeutic use of 2-ME may be deleterious in subjects with pre-established plaques; however, further in-depth studies are required to investigate this possibility. Finally, because recent findings indicate that some PPAR ligands are associated with significant increases in cardiovascular events among high-risk patients with type 2 diabetes mellitus,[28] it would be important to investigate whether 2-ME induces deleterious or beneficial actions as an activator of PPAR-associated genes.

Perspectives

2-ME is an investigational drug known to protect against multiple proliferative disorders, including vascular and renal diseases and hypertension. Microarray-based screening and identification of candidate genes modulated by 2-ME provide leads for better understanding the mechanisms via which 2-ME induces its vasoprotective and antivasoocclusive actions and help better define 2-ME's potential use as a therapeutic agent.

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Disclosures

None.

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