Peroxidase Properties of Extracellular Superoxide Dismutase: Role of Uric Acid in Modulating In Vivo Activity

H. Ulrich Hink, Nalini Santanam, Sergey Dikalov, Louise McCann, Andrew D. Nguyen, Sampath Parthasarathy, David G. Harrison, Tohru Fukai

Objective—The cytosolic form of Cu/Zn-containing superoxide dismutase (SOD1) has peroxidase activity, with H₂O₂ used as a substrate to oxidize other molecules. We examined peroxidase properties of the extracellular form of SOD (SOD3), a major isof orm of SOD in the vessel wall, by using recombinant SOD3 and an in vivo model of atherosclerosis.

Methods and Results—In the presence of HCO₃⁻, SOD3 reacted with H₂O₂ to produce a hydroxyl radical adduct of the spin trap DEPMPO. SOD1 and SOD3 were inactivated by H₂O₂ in a dose- and time-dependent fashion, and this was prevented by physiological levels of uric acid. To examine the in vivo role of uric acid on SOD1 and SOD3, control and apolipoprotein E–deficient (ApoE⁻/⁻) mice were treated with oxonic acid, which inhibits urate metabolism. This treatment increased plasma levels of uric acid in control and ApoE⁻/⁻ mice by ~3-fold. Although increasing uric acid levels did not alter aortic SOD1 and SOD3 protein expression, aortic SOD1 and SOD3 activities were increased by 2- to 3-fold in aortas from ApoE⁻/⁻ mice but not in aortas from control mice.

Conclusions—These studies show that SOD1 and SOD3 are partially inactivated in atherosclerotic vessels of ApoE⁻/⁻ mice and that levels of uric acid commonly encountered in vivo may regulate vascular redox state by preserving the activity of these enzymes. (Arterioscler Thromb Vasc Biol. 2002;22:

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Cyttoplasmic Cu/Zn superoxide dismutase (SOD1) not only catalyzes dismutation of O₂⁻ to H₂O, but also has peroxidase activity, with H₂O₂ used as a substrate, forming a copper-bound hydroxyl (OH) radical. Several lines of evidence suggest that this bound OH attacks ~1 adjacent histidine residue that binds copper, leading to loss of copper and inactivation of the enzyme. This inactivation of SOD1 by H₂O₂ may be prevented by coinubation with small anions or other reductants, such as formate, tocopherol, nitrite, and uric acid, resulting in the formation of their respective radicals. Recently, it has been recognized that HCO₃⁻ is important in this reaction, either by formation of the bicarbonate radical or by facilitating the reaction of H₂O₂ with Cu²⁺ in the active site of the enzyme.

Extracellular SOD (SOD3) is also a Cu/Zn-containing SOD that shares ~50% sequence homology with SOD1 within its catalytic region. Compared with the x-ray crystallography structure data for SOD1, all ligands to the copper and zinc atoms can be identified in SOD3, as can the cysteines forming the intramolecular disulfide bond. Thus, these findings suggest that the conformation of the active copper-binding site of SOD3 may resemble the active site of SOD1. The rate constants for dismutation of O₂⁻ by SOD1 and SOD3 are similar, and both enzymes are inhibited by cyanide, azide, and diethyldithiocarbamate. Because SOD3 constitutes 30% to 50% of the total SOD in vascular tissues and is localized in the extracellular space, it would be of considerable importance to determine whether SOD3 also has peroxidase activity and to determine which small molecule reductants can preserve its dismutase activity.

Although peroxidase properties of SOD1 have been studied extensively in vitro, evidence is lacking to prove that either SOD1 or SOD3 activities are affected by H₂O₂ in vivo. In the present study, we sought to determine whether SOD3 has peroxidase activities that are similar to those of SOD1 and to examine small molecules that might prevent the inactivation of SOD3 by using recombinant SOD3 expressed in Pichia pastoris. In these experiments, we found that physiological levels of uric acid completely prevented the inactivation of SOD1 and SOD3 by H₂O₂. In additional experiments, we provide evidence for peroxidase activity of SOD1 and SOD3 in vivo, by showing that these enzymes seem to be partially inactivated in the aortas of apolipoprotein E–deficient (ApoE⁻/⁻) mice and that the activity of these enzymes can be restored by elevating the endogenous levels of uric acid.

Materials and Reagents
All reagents were purchased from Sigma Chemical Co, including bovine Cu/Zn SOD (SOD1 and SOD3, 3000 to 5000 U/mg), unless otherwise stated.
otherwise noted. Chelex-100 resin was purchased from Bio-Rad. The pPIC9K-vector was purchased from Invitrogen. Steri-Vac filters and Prep-Scale concentrators were obtained from Millipore. CNBr-activated sepharose was obtained from Amersham Pharmacia Biotech. Catalase was purchased from Roche. Silver staining was performed by using a commercially available kit (Pierce). DEPMPO was obtained from Oxis International. Potassium phosphate buffers were prepared from KH₂PO₄ and K₂HPO₄ in the concentrations given. All buffers contained diethylenetriamine pentaaacetic acid (DTPA, 100 μmol/L) and were treated with chelex-100 (5 mg/100 mL, with gentle stirring for ≥1 hour) before use.

### Overexpression and Purification of Recombinant SOD3

The murine SOD3 was overexpressed in *P. pastoris*. The cDNA encoding SOD3, minus its original signaling domain, was cloned into the EcoRI and NotI sites of the pPIC9K vector, and this vector was transfected into the GS115 *P. pastoris* strain by electroporation. Large amounts of SOD3 were obtained by amplifying yeast in a basal fermentation media containing 160 mol/L copper and zinc with the use of methanol induction. The medium containing SOD3 protein was filtered (Steri-Vac GP-10) and concentrated with the use of a flow concentrator (2.5 ft², Prep-Scale TFF, MWCO 10,000). After 2 washings in 50 mmol/L sodium phosphate buffer (pH 7.8) to eliminate low molecular impurities including loosely bound copper, SOD3 was affinity-purified by an anti-SOD3 antibody linked to a CNBr sepharose column according to manufacturer’s instructions. The concentrated protein fraction was further purified by use of the Resource Q FPLC column (Pharmacia). The pooled SOD3 fractions were dialyzed in 50 mmol/L Tris/HCl, pH 7.5. The resulting sample was applied to a Resource Q FPLC column. The column was washed with 50 mmol/L Tris/HCl, pH 7.5, and eluted with a linear gradient of NaCl (0 to 1 mol/L) at 0.5%/min with a flow rate of 1 mL/min. Fractions containing SOD3 were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and pooled. The resultant purified protein yielded 2 bands on silver staining with molecular weights of 33 and 66 kDa, corresponding to monomeric and dimeric forms of the enzyme. SOD activity was measured in 50 mmol/L phosphate buffer by inhibition of the reduction of cytochrome c (50 μmol/L) by superoxide generated by xanthine (0.1 mmol/L) and xanthine oxidase (0.01 U/mL) at pH 7.8, as described previously. The specific activity of recombinant SOD3 was 2087 U/mg, similar to the previously purified SOD3. This activity was completely blocked by KCN (1 mmol/L).

### ESR Spectroscopy

Electron spin resonance (ESR) measurements were performed by using a Bruker EMX spectrometer at 9.78 GHz (ER 041 XG Microwave Bridge X-Band). DEPMPO was used as a spin trap. Spectrometer settings were as follows: microwave power 20.02 mW, time constant 163 ms, sweep time 83.89 seconds, center field 3480 G, sweep width 110 G, and modulation amplitude 1 G.

### Determination of Serum Uric Acid Levels

Serum uric acid levels were measured by using a quantitative enzymatic assay (Sigma) according to the manufacturer’s protocol, and the results were standardized by using standard solutions of uric acid (Sigma).

### Statistical Analysis

Data are presented as mean ± SEM. Western blots were quantified by use of densitometry. Comparisons between groups of mice were performed by using ANOVA, followed by a Dunnett ad hoc test. Values of P<0.05 were considered significant.

## Results

### Effect of H₂O₂ on SOD Activity and Formation of DEPMPO-OH Adduct

Because the initial steps of the peroxidase reaction led to inactivation of SOD1 in the absence of cosubstrates, we initially examined the ability of H₂O₂ to inactivate SOD3 compared with SOD1. The enzymes were incubated with H₂O₂ in 50 mmol/L phosphate buffer at pH 7.8. Both enzymes were inactivated to a similar extent by H₂O₂ in a time- and dose-dependent manner (Figure 1). The inactivation of these enzymes by H₂O₂ was not significantly affected by the addition of 23.5 mmol/L HCO₃⁻ (data not shown).

To determine whether SOD3 could catalyze the formation of a hydroxyl-like species on reaction with H₂O₂, we performed additional studies with use of the spin trap DEPMPO.
In the absence of HCO$_3^-$, incubation mixtures containing SOD3, DEPMPO, H$_2$O$_2$, and DTPA in 50 mmol/L phosphate buffer (pH 7.8) yielded no DEPMPO-OH (Figure 2). In the presence of HCO$_3^-$, however, the level of DEPMPO-OH adduct obtained on incubation of SOD3 with H$_2$O$_2$ was clearly increased. These data obtained with SOD3 are consistent with previous studies with SOD1, in which HCO$_3^-$ was found to have minimal effects on the inhibition of SOD1 activity by H$_2$O$_2$ but dramatically increased the hydroxylation of DEPMPO by SOD1 in the presence of H$_2$O$_2$.$^{5,16}$

### Cosubstrate Utilization by SOD1 and SOD3

Anionic radical scavengers, such as formate, urate, and azide, can prevent the inactivation of SOD1 by H$_2$O$_2$ because of the favorable electrostatic interaction.$^4$ To determine whether SOD3 shared this property, both SODs were exposed to various reductants during incubation with H$_2$O$_2$. Supraphysiological levels of nitrite (1 mmol/L) prevented the inactivation of SOD1 but only partially prevented the inactivation of SOD3 (Figure 3A). In contrast, physiological levels of urate ($\approx$500 $\mu$mol/L) almost completely prevented the inactivation of SOD1 and SOD3 by H$_2$O$_2$ (Figure 3B). Other potential substrates, including formate, glutamate, tyrosine, lactate, $\gamma$-tocopherol, histidine, arginine, and uracil, were not as effective as either nitrite or urate, even when applied in high concentrations (1 mmol/L, Figure 3C).

### In Vivo Evidence for Protection of SOD1 and SOD3 Activity by Uric Acid

Because uric acid is capable of preventing H$_2$O$_2$-induced inactivation of SOD1 and SOD3, we sought to determine whether increasing uric acid levels in vivo could increase the activity of these enzymes in the aortas of ApoE$^{-/-}$ mice. When the animals were maintained and studied at 6 to 8 months of age and even when they were fed a normal chow diet, the aortas of these animals developed extensive atherosclerosis and exhibited increased production of vascular reactive oxygen species.$^{17}$ We have previously shown that SOD3 protein levels are increased in their aortas.$^{14}$ We reasoned that the increase in H$_2$O$_2$ levels resulting from increased O$_2^-$ production may partially inactivate SOD1 and SOD3 in this model. To increase endogenous levels of uric acid, oxonic acid was infused by osmotic minipump at a rate of 2.8 mg/kg per day. Oxonic acid is a potent inhibitor of the enzyme uricase, which in mice is responsible for the metabolism of uric acid to allantoin.$^{18}$ A 7-day infusion of oxonic acid increased the plasma levels of uric acid by 3-fold in control and ApoE$^{-/-}$ mice (Figure 4). The activities of SOD1 and SOD3 were not altered by oxonic acid infusion in vessels from control animals (Figure 5A). In ApoE$^{-/-}$ mice, however, increased uric acid markedly increased the activities of SOD3 and SOD1 by 2.5±0.1- and 1.7±0.3-fold, respectively (Figure 5A). As previously shown,$^{14}$ the protein level of SOD3 but not SOD1 was higher in aortic vessels from ApoE$^{-/-}$ mice than in aortic vessels from control mice, but changing uric acid levels had no effect on either protein (Figure 5B).

We considered the possibility that oxonic acid might directly prevent SOD3 inactivation by H$_2$O$_2$ or might inhibit the production of H$_2$O$_2$ by enzyme systems in the vessel walls of ApoE$^{-/-}$ mice. In additional studies, we found that oxonic acid in concentrations up to 1 mmol/L had no effect on the inactivation of SOD3 by H$_2$O$_2$ (data not shown). Furthermore, we examined the effect of oxonic acid on the production of O$_2^-$ by cultured mouse aortic vascular smooth muscle cells. These cells were exposed to oxonic acid (100 $\mu$mol/L) for 24 hours, and the production of O$_2^-$ was determined by examining oxidation of the spin trap CP-H to CP by using electron spin resonance. Cells treated or untreated with oxonic acid produced identical levels of O$_2^-$ (data not shown). These data indicate that oxonic acid has no effect on the production of reactive oxygen species by vascular cells.

### Discussion

In the present study, we found that SOD3 has peroxidase properties that are very similar to those of SOD1. SOD3 is inactivated by H$_2$O$_2$ in a fashion similar to that of SOD1. In the presence of HCO$_3^-$, a DEPMPO-OH adduct was formed.
when SOD3 was exposed to H$_2$O$_2$, as has been shown previously for SOD1. In physiologically relevant concentrations, uric acid prevented this inactivation of SOD1 and SOD3, whereas other small molecules were much less effective, or they affected SOD activities only in supraphysiolog-ical concentrations. Importantly, we found that increased levels of uric acid in vivo rather dramatically increased the activity of SOD1 and SOD3 in the aortas of ApoE$^{-/-}$ mice but not in those of control mice. These findings provide the first evidence that peroxidase properties of SOD1 and SOD3 modulate the activity of these enzymes in vivo and provide a new mechanism whereby H$_2$O$_2$, by inactivating SOD, can worsen oxidative stress in the vessel wall.

Figure 3. Effect of various reductants on the H$_2$O$_2$-induced inhibition of SOD1 and SOD3. In the presence or absence of the indicated reductants (A, nitrite; B, uric acid; and C, various reductants as indicated), 2.5 U/mL of either enzyme was incubated with H$_2$O$_2$ (100 µmol/L) for 3.5 hours at 37°C. Thereafter, SOD activity was measured by cytochrome $c$ reduction assay in the presence of catalase (1000 U/mL) and DTPA (100 µmol/L). Data are expressed as the mean±SEM from at least 3 independent experiments performed in duplicate.

Figure 6 illustrates the peroxidase activity of SOD3 as demonstrated in the present studies. Inactivation of SOD3 by H$_2$O$_2$ likely involves the formation of either SOD-Cu·OH or another similar species in a fashion similar to that reported for SOD1.$^{19}$ Using the spin trap DEPMPO, we could readily observe the formation of a DEPMPO·OH adduct when SOD3 was exposed to H$_2$O$_2$. As has been reported for SOD1, formation of this adduct occurred only in the presence of the HCO$_3^-$ anion. Several studies have shown that the HCO$_3^-$ anion greatly enhances the peroxidase activity of SOD1.$^{4-6,16}$ Although there is some debate about the mechanisms involved, recent data support the concept that HCO$_3^-$, because of its negative charge and small size, readily enters the active
site of SOD1, where it is oxidized to the bicarbonate radical (CO$_3$·$^-$) by Cu$^{2+}$-OH, which was previously formed by the reaction of H$_2$O$_2$ with SOD1. The bicarbonate radical can readily diffuse out of the active site of SOD1 to react with other small molecules. In the case of nitron spin traps, such as DMPO and DEPMPO, the reaction with CO$_3$· leads to formation of their respective nitron radical cations, which then undergo hydrolysis to form DMPO-OH and DEPMPO-OH.

Figure 4. Effect of oxonic acid (OXO) treatment on plasma uric acid levels. Plasma uric acid levels (mg/dL) in control and ApoE$^{-/-}$ mice before and after OXO infusion with osmotic minipumps for 7 days ($n=4$). *$P<0.05$ between 2 groups.

Figure 5. Effect of oxonic acid on SOD1 and SOD3 activity and protein expression. Oxonic acid was infused in control (C57BL/6) and ApoE$^{-/-}$ mice for 7 days by osmotic minipumps. A, Aortas were homogenized, and SOD activity was assayed by examining the inhibition of cytochrome c reduction by xanthine/xanthine oxidase at pH 7.4. The activity of SOD3 was determined after separation with concanavalin A-sepharose. Experiments were performed on 4 pooled aortas on 3 separate occasions. The results were presented as mean±SEM, and the values were expressed as units per milligram of total protein. *$P<0.05$ vs control. B, Protein levels of SOD3 and SOD1 were determined by Western analysis (20 μg protein of homogenate from aortic tissue per lane). Representative blots are from 4 individual experiments.

Figure 6. Peroxidase activity of SOD 1 and SOD3. The active SOD3 reacts with H$_2$O$_2$ to form an intermediate (SOD3-OH radical) that can interact with the bicarbonate to ultimately lead to the formation of a DEPMPO-OH adduct. In the absence of a cosubstrate, SOD3 (SOD1) is inactivated by H$_2$O$_2$. In the presence of physiological levels of uric acid, however, SOD3 (SOD1) inactivation is prevented. The urate radical formed has low oxidative potential and may react with ascorbic acid to regenerate uric acid.
Our current findings suggest that the HCO$_3^-$ anion and its radical have a similar role in the peroxidase activity of SOD3. In the original characterization of SOD3, Marklund$^6$ showed that this enzyme was inactivated by H$_2$O$_2$. Our present study extends these observations by showing that this involves a peroxidase-like activity, with formation of a hydroxyl-like radical. In addition, a major goal of the present study was to determine which small molecules were effective in preventing H$_2$O$_2$ inactivation of SOD3. Although it is impossible to study all potential reductants, we focused on molecules previously reported to prevent the inactivation of SOD1 and, in particular, those present in vivo. Of note, in the present study, the HCO$_3^-$ anion did not prevent H$_2$O$_2$ inactivation of either enzyme. This result is in keeping with the recent finding of Liochev and Fridovich,$^6$ who showed that HCO$_3^-$ had no effect on the inactivation of SOD1. Likewise, Gross et al$^5$ have recently shown that HCO$_3^-$ minimally protects against the inactivation of SOD1 by H$_2$O$_2$. Several other potential substrates, including formate, glutamate, tyrosine, lactate, γ-tocopherol, histidine, arginine, and uracil, in concentrations exceeding those encountered in vivo, had minimal impact on the inactivation of either enzyme by H$_2$O$_2$. Of note, as reported previously, nitrite (1 mmol/L) prevented the inactivation of SOD1. This concentration of nitrite only partially prevented the inactivation of SOD3 by H$_2$O$_2$. It should be noted that the plasma concentrations of nitrite are markedly lower than this, ranging from 4.2 to 6.1 μmol/L.$^20$

Further experiments demonstrated that uric acid, in concentrations present in human plasma, was able to almost completely prevent the inactivation of SOD3 by H$_2$O$_2$ in a fashion similar to that demonstrated for SOD1 by Hodgson and Fridovich.$^1$ Importantly, the resulting urate radical likely formed in this process is relatively stable (redox potential 0.59 V at pH 7) and does not react with oxygen,$^21$ a property that contributes to the radical chain-breaking potential of urate. In human plasma, the urate radical can react readily with the weaker oxidant ascorbate, thereby regenerating urate and producing the even more innocuous ascorbate radical (Asc) with a redox potential of only 0.28 V.$^22$ Because normal human plasma concentrations of urate (125 to 500 μmol/L) are ~60-fold higher than those of nitrite,$^{20}$ the most likely product of the peroxidase reaction catalyzed by SOD3 in vivo is the relatively unreactive urate radical, which will be regenerated to urate by ascorbic acid. These findings indicate a new and potentially important antioxidant role of urate, aside from its known ability to react with potent oxidants such as peroxynitrite.$^{23}$

In a previous study,$^{14}$ we found that the protein expression of SOD3 is increased by as much as 3-fold in the aortas of ApoE$^{-/-}$ mice, seemingly from lipid-laden macrophages. In contrast to this 3-fold increase in protein expression, the activity of SOD3 was increased by only 2-fold. Our present findings provide some insight into this apparent discrepancy and suggest that SOD1 and SOD3 are partially inactivated in the aortas of ApoE$^{-/-}$ mice. By infusing oxonic acid, an inhibitor of uricase, we were able to increase uric acid levels by ~3-fold. In ApoE$^{-/-}$ mice, this increase in uric acid rather dramatically increased the vascular activity of SOD1 and SOD3, while having no effect on the amounts of protein of either of these enzymes as determined by Western analysis. Importantly, oxonic acid infusion had no effect on SOD1 or SOD3 activity in aortas from control mice, although it increased the plasma levels of uric acid to the same extent in control mice and in ApoE$^{-/-}$ mice. These findings suggest that in the presence of a pro-oxidant state such as atherosclerosis, these enzymes are partially inactivated. This phenomenon may contribute to the increased levels of O$_2^·$ observed in atherosclerotic vessels, because inactivation of these superoxide dismutases would reduce clearance of this radical.

Of interest, oxonic acid infusion in ApoE$^{-/-}$ mice caused a rather dramatic increase in SOD1 activity above that observed in control mice, without a change in SOD1 protein expression (Figure 5). The mechanisms responsible for this augmentation in SOD1 activity remain undefined but clearly reflect a posttranslational change in enzyme function. Recently, SOD1 activity in myeloid cells has been shown to be inhibited by phosphorylation in response to granulocyte colony-stimulating factor.$^{24}$ Whether phosphorylation of SOD1 is occurring in the setting of atherosclerosis remains unclear.

Uric acid is a potent scavenger of peroxynitrite; however, it is unlikely that its effect on SOD1 and SOD3 activities observed in the present study was due to the scavenging of peroxynitrite by uric acid. Previous studies from other laboratories$^{25}$ and our preliminary studies indicate that peroxynitrite does not alter the activity of either SOD1 or SOD3.

The role of uric acid in cardiovascular disease remains equivocal. Although some epidemiological studies have shown an independent association between elevated serum levels of uric acid and increased cardiovascular disease,$^{26-28}$ others have suggested that this association is confounded by the coexistence of conditions such as hypertension, obesity, hyperlipidemia, and diabetes mellitus, all known to be independent risk factors for atherosclerosis.$^{29-31}$ Interestingly, in most mammals, uric acid is further metabolized to allantoin by the enzyme uricase. In humans and higher primates, however, uric acid is the final product of purine metabolism.$^{32}$ It has been hypothesized that uric acid may be an evolutionary antioxidant scavenge/ for the loss of the ability to synthesize ascorbate in higher primates.$^{33}$ Indeed, in vitro experiments have demonstrated that uric acid is an effective scavenger of free radicals$^{33}$ and can chelate transition metal ions.$^{34}$ Paradoxically, uric acid has also been reported to increase oxidative damage,$^{35}$ to enhance platelet adhesive- ness,$^{36}$ and to stimulate vascular smooth muscle cell growth.$^{37}$ Hence, the overall functional importance of urate in vivo remains unknown. Our present data suggest that one potential beneficial effect of uric acid may relate to its ability to prevent the inactivation of SOD1 and SOD3 by H$_2$O$_2$. In humans, the levels of uric acid normally encountered in the Western countries are generally high enough to prevent H$_2$O$_2$ inactivation of either SOD1 or SOD3. Of note, levels of uric acid may be decreased in Fanconi’s syndrome,$^{38}$ in the acquired immunodeficiency syndrome,$^{39}$ during space flight,$^{40}$ in type II diabetes,$^{41}$ and by allopurinol treatment. In these conditions, it is conceivable that H$_2$O$_2$ inactivation of SOD1 and SOD3 may occur and contribute to oxidant stress.
Because other H₂O₂-metabolizing enzymes, such as glutathione peroxidase and catalase, are located intracellularly, it is possible that SOD3 has a predominant role in the disposal of H₂O₂ in the extracellular space. This may have particular importance in vascular homeostasis. We have previously shown that vascular expression of SOD3 seems to be highly regulated in pathophysiological and physiological conditions, including atherosclerosis, and angiotensin II–induced hypertension, and exercise training. It is interesting to speculate that under these conditions, increased SOD3 expression not only reduces interstitial O₂⁻ but also minimizes interstitial H₂O₂ levels.

In summary, these studies demonstrate that SOD3 has peroxidase properties that are similar to those of SOD1. We further show that physiological levels of uric acid importanty modulate the activity of SOD1 and SOD3 by preventing the inactivation of these enzymes by H₂O₂. In the setting of increased vascular oxidant stress, as encountered in atherosclerotic vessels, SOD1 and SOD3 are partially inactivated, and this can be prevented by increasing the circulating levels of uric acid within the physiological range. These data show that the inactivation of SOD by H₂O₂ may represent a new mechanism contributing to oxidant stress in vivo, and they also show an important role of uric acid in modulating this phenomenon.

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