

Methylglyoxal Induces Prostaglandin E₂ Production in Rat Mesangial Cells

ATSUKO URIUHARA, SATOSHI MIYATA, BING-FEN LIU,
HIROYUKI MIYAZAKI, HITOMI KUSUNOKI, HIROSHI KOJIMA,
YUMIKO YAMASHITA, KOTARO SUZUKI, KAYO INABA,
and MASATO KASUGA

*Division of Diabetes, Metabolism, and Endocrinology, Department of Internal Medicine,
Kobe University Graduate School of Medicine, Kobe, Japan*

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The formation of methylglyoxal (MG), a reactive dicarbonyl compound, is accelerated under hyperglycemia, presumably contributing to tissue injury in diabetes. On the other hand, prostaglandin E₂ (PGE₂) has been implicated in glomerular hyperfiltration, a characteristic change in the early stage of diabetic nephropathy. We therefore examined whether MG was capable of inducing PGE₂ production in rat mesangial cells (RMC) to address a possible mechanism by which hyperglycemia-derived dicarbonyls accelerated the development of diabetic nephropathy. RMC were incubated with 0 - 200 μM of MG, followed by determination of secreted PGE₂ by enzyme immunoassay (EIA). We further investigated the intracellular mechanisms mediating the MG-induced PGE₂ synthesis, focusing particularly on cyclooxygenase-2 (COX-2) and the MAPK superfamily. Our results indicated that MG induced PGE₂ production in a dose-dependent manner, accompanied by augmentation of COX-2 mRNA expression. This MG-induced PGE₂ production was significantly suppressed by inhibiting either ERK1/2 or p38 MAPK, implicating involvement of the MAPK superfamily. Our results suggest a potential role of MG in the development of diabetic nephropathy through PGE₂ production, and may serve as a novel insight into the therapeutic strategies for diabetic nephropathy.

Persistent hyperglycemia has been established as the underlying cause of diabetic microangiopathy, including diabetic nephropathy(6). One of the mechanisms of hyperglycemia-induced tissue damage in diabetic nephropathy is the glycation reaction, since several types of advanced glycation end-products (AGEs) have been shown to localize in the glomeruli of diabetic subjects (19,38). Reactive dicarbonyl intermediates, such as 3-deoxyglucosone (3-DG) and methylglyoxal (MG) are potent precursors of AGEs (12,17, 41,47). The formation of these dicarbonyls is thought to be accelerated through several pathways under diabetic conditions. For example, MG can form from triose phosphates that are intermediates of the glycolytic pathway (43), in addition to the pathway through the glycation reaction. Indeed, evidence for the increase in levels of 3-DG and MG in diabetic subjects has been accumulating (2,26,35,53). Furthermore, recent studies have suggested that these dicarbonyl compounds, *per se*, play an important role in the pathogenesis of diabetic complications, since they are highly reactive in modulating the structure and function of

intracellular proteins (8,13,31,46). However, the involvement of the dicarbonyl compounds in the development of diabetic nephropathy has not been fully investigated.

On the other hand, glomerular filtration rate (GFR) is elevated in the early phase of diabetes (11,20). This alteration in renal hemodynamics causes intraglomerular hypertension, leading to further glomerular injury (39). In this respect, the vasodilatory prostaglandins, including PGE₂, have been implicated in the glomerular hyperfiltration seen in diabetic patients (11,14,18,51), as well as in experimental models of diabetes (9,10,22). Barnett *et al.* (1) reported that glomeruli isolated from streptozotocin-induced diabetic rats, with 3 weeks of diabetes onset, produce significantly more PGE₂ than those from control rats. However, the precise mechanism of PGE₂ overproduction in diabetic renal tissue remains unclear.

The present study was designed to investigate the role of hyperglycemia-derived dicarbonyl compounds in the development of diabetic nephropathy by specifically determining whether MG can induce PGE₂ production in rat mesangial cells. We also investigated the mechanisms by which MG induces the expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme in PGE₂ biosynthesis. In particular, we focused on MAPK superfamily signaling, which has recently been postulated to mediate COX-2 activation in a variety of cells (16,27,29,34,40).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle medium (DMEM) containing low (1 g/l) glucose, Dulbecco's phosphate-buffered saline (D-PBS) and penicillin/streptomycin were purchased from GIBCO, BRL (Rockville, MD). Fetal calf serum (FCS) was purchased from the JRH Biosciences CSL Company (Lenexa, KS). MG and aminoguanidine were purchased from the Sigma Chemical Co. (St. Louis, MO). L-NAME was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). NS-398 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). All other chemicals were also of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise noted.

Cell culture. Rat mesangial cells (RMC) were obtained from the glomeruli of renal cortex isolated from male Sprague-Dawley rats (CLEA Japan, Inc., Osaka) as described previously (25). The cells were maintained in DMEM supplemented with 17% FCS, penicillin (100 µg/ml) and streptomycin (100 µg/ml) at 37°C under 5% CO₂ atmosphere. Cells between passages 4 and 6 were used for the following experiments. All animal experiments were conducted according to the "Guidelines for Animal Experimentation" at Kobe University School of Medicine.

Experimental cell treatments. When RMC reached approximately 80% confluence in 24-well tissue culture plates, they were starved in DMEM with 2% FCS for 2 days, followed by two washes in D-PBS. To examine the effects of MG on PGE₂ production, the cells were incubated with 0, 10, 100 or 200 µM MG in D-PBS for 0 - 8 h. After incubation, the amount of PGE₂ secreted into the culture medium was determined using the PGE₂ EIA kit (Cayman Chemical Company, Ann Arbor, MI). All assays were performed in triplicate. We obtained representative results in each experiment by performing several separate assays to confirm reproducibility.

For the inhibition experiments, the cells were incubated with 200 µM MG for 4 h in the presence or absence of aminoguanidine (AG; 50,100 or 200 µM), a glycation inhibitor, or L-NAME (50,100 or 200 µM), an authentic inhibitor of inducible nitric oxide synthase (iNOS). In addition, we examined the effects of NS-398, a selective inhibitor of COX-2, on MG-induced PGE₂ production. RMC were treated with NS-398 (0.1, 1 or 10 µM) from 2 h

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prior to incubation with MG. After 2 and 4 h, PGE₂ secreted into the supernatants was determined as described above.

Northern blot analysis. RMC were incubated in the presence or absence of 200 μ M MG following 2 days starvation as described above. After 2 h, total RNA was extracted from the cells using the Total RNeasy mini kit (Qiagen K.K., Tokyo). Each total RNA sample (14 μ g/lane) was analyzed by Northern hybridization with a rat COX-2 cDNA probe (Oxford Biomedical Research, Inc. Oxford, MI) which was labeled using Gene Images Random-Prime Labeling Module (Amersham Biosciences Corp., Piscataway, NJ). RNA levels were standardized to the 28S RNA band stained with ethidium bromide.

Involvement of the MAPK superfamily in PGE₂ production. We investigated whether the MAPK superfamily, including ERK1/2 and p38 MAPK, is involved in MG-induced PGE₂ production by using selective inhibitors against these molecules. First, we evaluated the MG-induced phosphorylation of both ERK1/2 and p38 MAPK in RMC. Briefly, RMC were preincubated with or without inhibitors such as PD98059 (30 μ M), an inhibitor of ERK1/2 and SB203580 (10 μ M), an inhibitor of p38 MAPK, for 1 h. The cells were subsequently incubated with 200 μ M MG for another hour, washed with ice-cold PBS and then lysed in 100 μ l of SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue]. Cell lysates were passed through 21-gauge needles on ice several times and then electrophoresed on a 10% SDS-polyacrylamide gel. The separated protein bands were transferred onto a nitrocellulose membrane and subjected to immunoblot analysis using a PhosphoPlus p44/42 (Thr202/Tyr204) Antibody Kit and PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) Antibody Kit (Cell Signaling Technology, Inc., Beverly, MA). The positive bands were visualized using a chemiluminescence detection system. Next, we investigated whether inhibition of phosphorylation of ERK1/2 or p38 MAPK affected the MG-induced PGE₂ production in RMC. After the same treatment as above, RMC were incubated with MG for 2 or 4 h. The PGE₂ concentration in the supernatant was determined as described above.

Statistical analysis. Data from the PGE₂ assay were expressed as mean \pm SD and were analyzed by one-way ANOVA followed by the Dunnett multiple comparison test. Differences between groups were deemed statistically significant when P values were less than 0.05.

RESULTS

MG-induced PGE₂ production in RMC. MG induced PGE₂ production in RMC in time- and dose-dependent manners (Fig. 1). At 8 h of incubation, PGE₂ production was significantly ($p < 0.01$) enhanced in RMC incubated with more than 100 μ M MG, compared with that in RMC incubated without MG. AG inhibited the MG (200 μ M)-induced PGE₂ production in a dose-dependent manner, such that the extent of inhibition became significant ($p < 0.05$) at the concentration of 100 μ M (Fig. 2). On the other hand, L-NAME, an authentic iNOS inhibitor, failed to suppress MG-induced PGE₂ production at the same concentration as AG. Treatment with higher concentrations of AG, such as 200 μ M, resulted in more significant ($p < 0.01$) suppression of the MG-induced PGE₂ production, while L-NAME caused less significant ($p < 0.05$) reduction (Fig. 2). Furthermore, we found that NS-398, a COX-2 selective inhibitor, also significantly inhibited MG-induced PGE₂ production by RMC in a dose-dependent manner (Fig. 3).

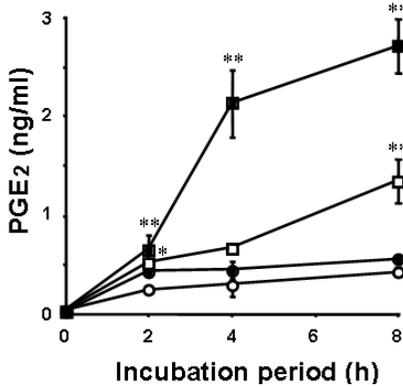


FIG. 1. RMC were incubated with 0 (open circles), 10 μM (closed circles), 100 μM (open squares) or 200 μM (closed squares) methylglyoxal (MG) for 2, 4 or 8 h. PGE₂ secreted in the supernatant was determined by EIA. MG induced PGE₂ production in a dose- and time-dependent manner. Data are mean \pm SD. * $p < 0.05$ and ** $p < 0.01$ vs. MG 0 μM .

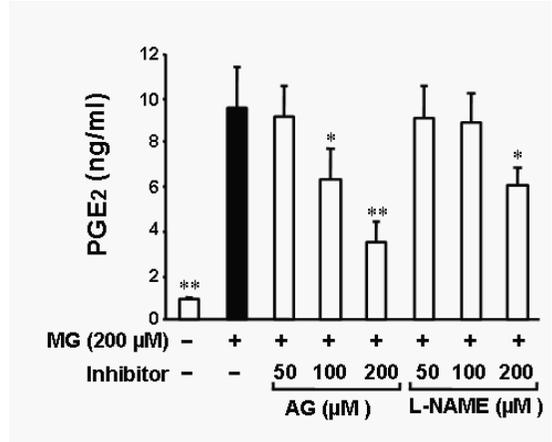


FIG. 2. RMC were incubated with 200 μM of MG in the absence or presence of either AG or L-NAME for 4 h. Each supernatant was then collected and assayed for PGE₂ content by EIA. AG suppressed the MG-induced PGE₂ synthesis in a dose-dependent manner, while L-NAME inhibited it to a lesser extent. Data are mean \pm SD. * $p < 0.05$ and ** $p < 0.01$ vs. MG 200 μM .

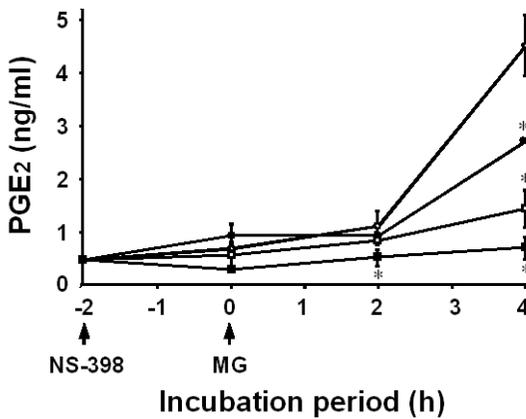


FIG. 3. RMC were pretreated with 0 (open circles), 0.1 (closed circles), 1 (open squares) or 10 μM (closed squares) of NS-398, a selective COX-2 inhibitor, for 2 h, followed by incubation with 200 μM MG. Supernatants were collected at 2 and 4 h for determination of PGE₂ concentration by EIA. NS-398 inhibited the MG-induced PGE₂ production in RMC in a dose-dependent manner. Data are mean \pm SD. * $p < 0.05$ and ** $p < 0.01$ vs. MG alone.

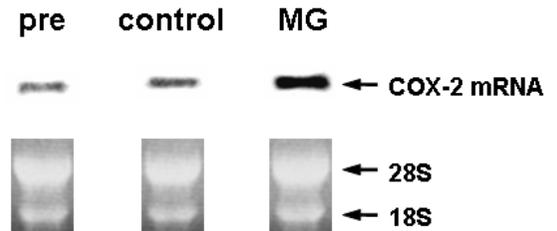


FIG. 4. Total RNA was extracted from mesangial cells incubated in the presence or absence of 200 μM MG for 2 h, and analyzed by Northern hybridization with a rat COX-2 cDNA probe. RNA levels were standardized to 18S and 28S RNA bands stained with ethidium bromide. The COX-2 mRNA level was increased in RMC incubated with MG, but not in RMC without MG incubation.

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Increased COX-2 mRNA expression induced by MG in RMC. Northern blot analysis revealed that COX-2 mRNA expression was increased in RMC when incubated with MG for 2 h, whereas no significant alteration of COX-2 mRNA levels was observed in RMC incubated without MG (Fig. 4).

Involvement of MAPK superfamily in MG-induced PGE₂ production. Immunoblot analysis revealed that incubation of RMC with 200 μ M MG resulted in phosphorylation of both ERK1/2 (Fig. 5A) and p38 MAPK (Fig. 5B) without a change in protein content of either molecule. PD98059 (30 μ M) efficiently suppressed the MG-induced ERK1/2 phosphorylation almost to control level, with little effect on p38 MAPK phosphorylation. Conversely, SB203580 (10 μ M) suppressed MG-induced p38 MAPK phosphorylation, without affecting ERK1/2 phosphorylation. With reference to the effects of these inhibitors on PGE₂ production, the MG (200 μ M)-induced PGE₂ production was significantly ($p < 0.01$) suppressed when RMC were treated with either PD98059 or SB203580 (Fig. 6). These results indicated that the extent of PGE₂ suppression by these inhibitors paralleled their respective inhibitory effects on phosphorylation of ERK1/2 or p38 MAPK.

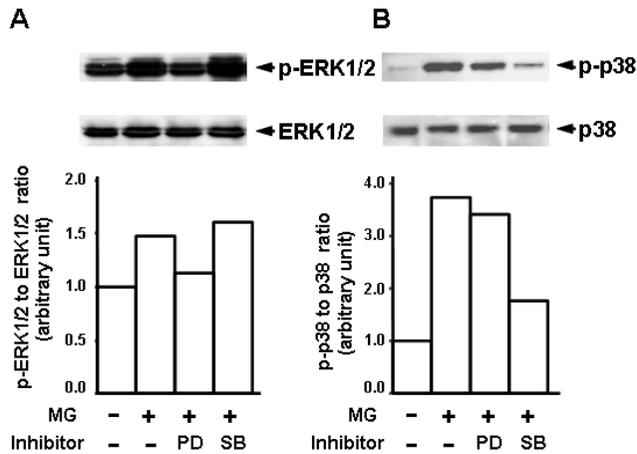


FIG. 5. RMC were preincubated with or without inhibitors such as PD98059 (PD; 30 μ M), an inhibitor of ERK1/2, and SB203580 (SB; 10 μ M), an inhibitor of p38 MAPK for 1 h, followed by incubation with 200 μ M of MG for another hour. The phosphorylated fraction and total protein of ERK1/2 (A) and p38 MAPK (B) in the cells were determined by immunoblot analysis. The phospho-ERK1/2 to total ERK1/2 ratio and phospho-p38 MAPK to total p38 MAPK ratio were calculated from the quantified intensities of the respective bands by NIH imaging. In each case, the ratio in RMC incubated without MG was expressed as 1. MG induced phosphorylation of both ERK1/2 and p38 MAPK, which was inhibited by the respective specific inhibitors without appreciable cross-reaction.

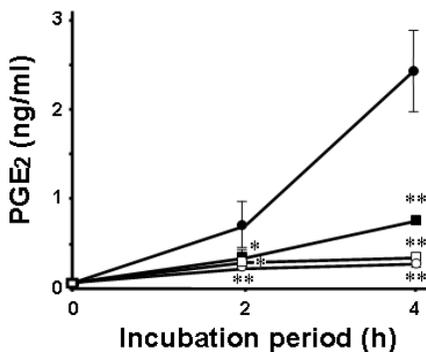


FIG. 6. RMC were preincubated in the absence or presence of the inhibitors PD98059 (30 μ M) or SB203580 (10 μ M) for 1 h, followed by incubation with 200 μ M MG. After 2 or 4 h, PGE₂ secreted in the supernatant was determined by EIA. Data for medium alone (open circles), MG without inhibitors (closed circles), MG with PD98059 (open squares) and MG with SB203580 (closed squares) were

plotted. Pretreatment of RMC with these inhibitors resulted in significant suppression of the MG-induced PGE₂ production. Data are mean ± SD. * p < 0.05 and **p < 0.01 vs. MG alone.

DISCUSSION

The present study indicated that MG was capable of inducing PGE₂ production in the mesangial cells in a dose-dependent manner. Formation of MG is accelerated under diabetic conditions due to hyperglycemia-related metabolism (2,35,42). It has been reported that plasma MG level is approximately 200 nM ~ 2 μM in diabetic rats (42) and patients (2,35); whereas the MG level in the kidney tissue is about sixfold higher (42,49). It is also known that only a few percent of the exogenous MG was taken into cells *in vitro* (8,44). Taken all these facts into account, the deduced intracellular concentration of MG in the cells incubated with 200 μM MG as performed in this study may be comparable to *in vivo* process. Furthermore, Lapolla *et al.* (28) recently described a possibility that plasma MG level reached the range around 400 μM by a new GC/MS method. We therefore consider that the MG concentration used in the present experiments is of physiological relevance. Thus, it is conceivable that the accelerated formation of MG is one of the mechanisms for the enhanced PGE₂ production in the diabetic kidney, as previously reported (1,9,10,11,14,18,22,51).

Our results also showed that aminoguanidine (AG) efficiently suppressed MG-induced PGE₂ production in RMC. This inhibitory effect may be attributable to chemical trapping of MG, since MG is efficiently scavenged by AG *in vitro* (50). However, AG has also been reported to exert an inhibitory action against iNOS (36,52). Therefore, we compared the inhibitory effect of AG with that of L-NAME, an authentic iNOS inhibitor. Our results showed that the inhibitory effect of AG overcame that of L-NAME, despite the fact that the IC₅₀ for iNOS of L-NAME (20-25 μM) (36) was even lower than that of AG (45 μM) (37), indicating that direct trapping of MG may be a crucial mechanism for the inhibitory effect of AG. In addition, our data showing a moderate inhibitory effect of L-NAME on MG-induced PGE₂ production may imply that MG stimulates PGE₂ production partly through NO synthesis, since NO has been reported to potentially induce PGE₂ production in certain cells (15,45). Although further studies are required to investigate whether MG can induce NO synthesis in RMC to clarify the involvement of NO, a report by Chang *et al.* (7) that MG induces significant generation of NO in rat vascular smooth muscle cells may imply a possible similar phenomenon in mesangial cells.

A recent immunohistochemical study showed that immunoreactive COX-2 was increased in renal tissue in diabetic rats (23,24). In the present study, MG-induced PGE₂ overproduction was concomitant with the increased COX-2 mRNA expression in RMC. Furthermore, NS-398, a selective COX-2 inhibitor, inhibited the MG-induced PGE₂ production in a dose-dependent manner. Taken together, these findings indicate that COX-2 is involved in MG-induced PGE₂ production. Recently, the MAPK superfamily, including molecules such as ERK1/2 and p38 MAPK, has been reported to mediate COX-2 activation in a variety of cells (16,27,29,34,40). Our findings also indicated the involvement of the MAPK superfamily in PGE₂ production in RMC. In the present study, MG-induced PGE₂ production was inhibited to almost control level by either PD98059 or SB203580 alone, suggesting that the ERK1/2 and p38 MAPK pathways may coordinately act to activate COX-2. This hypothesis may be supported by similar phenomena observed in other types of cells in response to different stimulants. For example, Matsuura *et al.* (34) showed that both ERK and p38 MAPK pathways were required for the increase in COX-2 mRNA level induced by transforming growth factor (TGF)-α in normal human epidermal keratinocytes. They suggested that the p38 MAPK signaling pathway controls COX-2 at the level of

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mRNA stability, while the ERK signaling pathway regulates COX-2 at the level of transcription. Furthermore, Guo *et al.* (16) have demonstrated the possibility that the ERK1/2 and p38 MAPK pathways independently contribute to AP-1 activation at the COX-2 promoter by induction of c-fos and c-jun, respectively, as a mechanism by which bombesin stimulates COX-2 expression in intestinal epithelial cells. In addition, Kiritoshi *et al.* (23) showed an involvement of NF- κ B activation through the enhanced production of reactive oxygen species (ROS) from the mitochondrial electron transport chain in the increased COX-2 expression and PGE₂ production in human mesangial cells cultured under high glucose concentration. Since putative binding motifs for both AP-1 and NF- κ B have been demonstrated at the upstream of COX-2 gene (32), COX-2-regulated PGE₂ production in glomeruli seems to be amplified by various mechanisms under diabetic conditions.

In addition to the activation of COX-2, p38 MAPK has been known to mediate a variety of cellular responses including apoptosis. We have previously reported that MG also induced apoptosis through activation of p38 MAPK in the same cell species used in the present study (31). We therefore evaluated cell viability by a trypan blue dye exclusion assay in the mesangial cells incubated with MG. As a result, the cell viability was slightly reduced by incubation with 200 μ M MG compared with medium alone (80.0 % vs. 86.9 % at 4h, and 75.0% vs. 76.7% at 8h). This reduction seems to be partly by apoptosis as we have demonstrated previously (31). We thus hypothesize that activation of p38 MAPK by MG accelerated PGE₂ production until the cell reaches apoptosis, since apoptosis is an ultimate response against any kind of cellular stresses.

Excess PGE₂ production has been suggested to play a role in the pathological renal hemodynamics and structural alterations in diabetic renal tissues (11,20). PGE₂ is known to modulate renal hemodynamics and salt and water excretion via four subtypes of E-prostanoid (EP) receptors. Through the EP4 receptor, a major subtype distributed in the glomerulus, PGE₂ reduces the contractility of mesangial cells and dilates the afferent arteriole (4,5,48), probably contributing to an increase in the GFR that is a hallmark of the early stage of diabetic nephropathy. In addition, PGE₂ reportedly stimulates renin secretion in the mouse renal juxtaglomerular apparatus (21). Given the accumulating evidence for an important role of the local renin-angiotensin system in development of diabetic nephropathy (3,30), PGE₂ may be one of the mediators activating this system. Furthermore, the recent study of Makino *et al.* (33) showed the preventive effect of an orally administered EP1 antagonist on glomerular hypertrophy and proteinuria in diabetic rats, thus implicating the EP1 subtype in diabetic nephropathy.

In conclusion, we demonstrated in the present study that MG induced PGE₂ production through upregulation of COX-2, at least in part mediated by ERK1/2 and p38 MAPK signaling pathways. This PGE₂ overproduction may result in disordered hemodynamics and pathological changes in the renal tissue, contributing to the development of diabetic nephropathy. These results may serve as a novel insight into therapeutic strategies for diabetic nephropathy, since these mediators are potential pharmacological targets.

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