Epigallocatechin Gallate, a Constituent of Green Tea, Represses Hepatic Glucose Production*

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For centuries, folk medicine has employed plants and herbs for their medicinal and protective abilities. Recent epidemiologic research shows a positive correlation between the consumption of fruits, vegetables, grains, and legumes and the prevention of chronic illnesses. Phytochemicals, naturally occurring plant biochemistry that give plants their color and flavor, may improve or prevent a number of chronic diseases because of their anti-inflammatory, antiatherosclerotic, antioxidant, and anticarcinogenic activity (1). Polyphenols, which include more than 4000 identified flavonoids, comprise one of the largest groups of active phytochemicals (2).

Green tea, a beverage commonly consumed in Asian countries, is a significant source of a type of flavonoids called catechins. The green tea catechins include (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate, and (−)-epicatechin (Fig. 1) (3). EGCG is the most abundant of these catechins, and many healthful benefits, including anticarcinogenic, antioxidant, antiangiogenic, and antiviral activities, have been ascribed to EGCG (4–7). EGCG may also possess antidiabetic activity. In a recent report, injection of EGCG into lean and obese Zucker rats significantly lowered blood glucose and insulin levels, and green tea extract increased glucose metabolism in adipocytes (8, 9). Additionally, (−)-epicatechin, which is structurally similar to EGCG, is the active compound in the extract of Pterocarpus marsupium Roxb bark, which is traditionally used in Indian folk medicine to treat diabetes (10).

One of the hallmarks of diabetes is the inability of insulin to inhibit hepatic glucose production. It has been suggested that increased gluconeogenesis is a main source of increased hepatic glucose production and that the ability of insulin to regulate transcription of the rate-controlling gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), may contribute to this problem. This point is underscored by the observation that in several animal models of type II diabetes and obesity, PEPCK mRNA levels are increased 2–3-fold over those observed in non-diabetic animals, despite the higher circulating insulin levels observed in the diabetic animals (11–13). Also, transgenic mice that overexpress PEPCK display a diabetes-like syndrome (14).

The rate of transcription of the hepatic PEPCK gene is increased by several hormones, including glucocorticoids, retinoic acid, and glucagon (via its second messenger, cAMP) (15–18). Insulin dominantly represses PEPCK gene transcription (19–21). The use of specific kinase inhibitors revealed that PI3K, but neither MAPK nor p70s6k, is involved in the insulin response of the PEPCK gene (22). A variety of other agents is insulinomimetic in the sense that these compounds reduce PEPCK mRNA levels. Such compounds include phorbol esters, compounds that elicit oxidative and cellular stress (such as H2O2 and sodium arsenite), and the cytokines tumor necrosis factor-α, interleukin-6, and interleukin-1. These agents differ from insulin, however, in that they repress PEPCK gene transcription in a PI3K-independent manner (20, 23–26).

Vanadate, a potent protein-tyrosine phosphatase inhibitor, also mimics several of the metabolic actions of insulin. For instance, vanadate lowers blood glucose in streptozotocin-induced diabetic rats, inhibits lipolysis in adipocytes, and inhibits phosphomonoesterase; P13K, phosphoinositide 3-kinase; PKB, protein kinase B; DMEM, Dulbecco’s modified Eagle’s medium; PTP, protein-tyrosine phosphatase; DCFH, 2’,7’-dichlorofluorescein diacetate; DCF, 2’,7’-dichlorofluorescein; IRS-1, insulin receptor substrate-1; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; Dex, dexamethasone; NAC, N-acetylcysteine; SOD, superoxide dismutase; ROS, reactive oxygen species; IR-β, β-subunit of the insulin receptor; MAPK, mitogen-activated protein kinase; MOPS, 4-morpholinepropanesulfonic acid.

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‡ The abbreviations used are: EGCG, (−)-epigallocatechin gallate; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; P13K, phosphoinositide 3-kinase; PKB, protein kinase B; DMEM, Dulbecco’s modified Eagle’s medium; PTP, protein-tyrosine phosphatase; DCFH, 2’,7’-dichlorofluorescein diacetate; DCF, 2’,7’-dichlorofluorescein; IRS-1, insulin receptor substrate-1; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; Dex, dexamethasone; NAC, N-acetylcysteine; SOD, superoxide dismutase; ROS, reactive oxygen species; IR-β, β-subunit of the insulin receptor; MAPK, mitogen-activated protein kinase; MOPS, 4-morpholinepropanesulfonic acid.
creases glucose transport into L6 myotubes (27–31). Unlike insulin, however, the above-listed effects of vanadate are independent of PI3K activity whereas the effects of insulin are PI3K-dependent (30, 31). Vanadate may act in vivo by enhancing insulin sensitivity and prolonging insulin action, effects that seem to be related to protein-tyrosine phosphatase (PTP) inhibition (32). Furthermore, vanadate directly inhibits the activity of two key gluconeogenic enzymes, PEPCK and G6Pase, which also contributes to decreased blood glucose levels in diabetic animals (33, 34).

The above-listed observations reveal that, although many diverse signals regulate glucose metabolism, an understanding of these signaling pathways should aid in the development of pharmacological agents to treat diabetes. A suitable antidiabetic agent should have actions similar to insulin, or it should bypass the defects in insulin action characterized by insulin resistance. Since EGCG reduces blood glucose by an unknown mechanism, the purpose of this study is to examine the effect of green tea compounds on insulin signaling pathways, gene expression, and glucose production. Our experiments reveal that EGCG has some insulinomimetic activities in hepatoma cells and that it differs from many other identified repressors of G6Pase, which also contributes to decreased blood glucose levels in diabetic animals (33, 34).

In contrast to insulin, however, the metabolic effects of these signaling pathways should aid in the development of pharmacological agents to treat diabetes. A suitable antidiabetic agent should have actions similar to insulin, or it should bypass the defects in insulin action characterized by insulin resistance. Since EGCG reduces blood glucose by an unknown mechanism, the purpose of this study is to examine the effect of green tea compounds on insulin signaling pathways, gene expression, and glucose production. Our experiments reveal that EGCG has some insulinomimetic activities in hepatoma cells and that it differs from many other identified repressors of G6Pase, which also contributes to decreased blood glucose levels in diabetic animals (33, 34).

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**EXPERIMENTAL PROCEDURES**

*Glucose Production Assay—*H4IIE rat hepatoma cells were treated with a combination of 500 nM dexamethasone and 0.1 mM 8-(4-chlorophenylthio)-cAMP in the presence or absence of insulin (Sigma-Aldrich) or EGCG (Sigma-Aldrich), for 5 h at 37 °C. Cells were incubated for an additional 3 h in glucose production buffer (glucose-free Dulbecco’s modified essential medium, pH 7.4, containing 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red) with dexamethasone and 0.1 mM 8-(4-chlorophenylthio)-cAMP in the presence or absence of insulin or EGCG. At the end of this incubation, 0.5 ml of medium was taken to measure the glucose concentration in the culture medium using a glucose assay kit (Sigma 510-A) (35). Cells were collected and lysed, and the total protein concentration was measured (Bio-Rad) to correct for cell count.

**Primer Extension and Ribonuclease Protection Assays—**Total RNA was isolated with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) using the instructions provided by the manufacturer. The PC28 and ACT25 oligonucleotides, which are complimentary to the mRNAs of the rat PEPCK and β-actin genes at positions 102–129 and 42–67, were used in primer extension assays as described previously (22). Ribonuclease protection assays were performed according to the instructions provided with the Ambion (Austin, TX) RPAII kit, as described previously (35). The rat glucose-6-phosphatase RNA probe was generated from polymerase chain reactions in which the downstream primer contained the T7 promoter. A 5-µl aliquot of the polymerase chain reaction was added directly to the components of the Ambion MaxiScrip kit, with [α-32P]UTP to produce radiolabeled RNA.

**Immunoprecipitation and Immunoblot Analysis—**H4IIE or HepG2 hepatoma cells were grown to confluence in Dulbecco’s modified Eagle’s medium (DMEM) containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum. H4IIE cells were serum-deprived for 24 h and then incubated in serum-free DMEM in the presence or absence of 10 nM insulin or various concentrations of EGCG. After different time points, cell extracts were prepared with a detergent lysis buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 20 mM NaP2O7, 0.2% Triton X-100, 10 nM microcystin, 0.1% β-mercaptoethanol, 0.1 mM phenylmethysulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin). Lysates were adjusted to 1 ml in lysis buffer before addition of the specific antisera used to immunoprecipitate the β-subunit of the insulin receptor (IR-β), IRS-1, or IGF-1R. The immunoprecipitations were carried out in reactions containing 2.5 µg of cell lysate and 2.5 µg of antiserum for 1.5 h at 4 °C. Protein A- or protein G-Sepharose beads (25 µl) were then added for an additional 1.5-h incubation followed by two washes of the immunoprecipitates in lysis buffer. The washed immunoprecipitates were dissolved in 45 µl of SDS sample buffer, boiled for 3 min, and proteins (30 µg per lane) were resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with specific antibodies for 2 h, followed by incubation with an anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (1:5000). Immunoreactive proteins were detected using the ECL immunoblotting system obtained from Amersham Biosciences, according to the manufacturer’s instructions. Alternatively, cell lysates were prepared as described above and directly dissolved in SDS sample buffer for analysis of proteins (PRB, p70S6K, and MAPK) by immunoblot analysis.
The production of glucose in response to hormones in both a physiological and consistent manner. The cells were treated with a combination of 500 nM dexamethasone and 1 mM 8-(4-chlorophenylthio)-cAMP (Dex/cAMP) in the presence or absence of insulin or EGCG, for 5 h. The cells were washed twice with phosphate-buffered saline and then were incubated in glucose-free DMEM, pH 7.4, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate for 3 h in the presence of Dex/cAMP with or without insulin or EGCG. The glucose concentration was measured in the extracellular medium as described under “Experimental Procedures.” Results are presented as percentages relative to the glucose produced by Dex/cAMP-treated H4IIE cells (100%). Data represent the mean of three experiments ± S.E., (*p < 0.05, Student’s t test).

Similar inhibition of glucose production was observed in hepatocytes after EGCG treatment but not after insulin treatment (data not shown). Others have shown that insulin does not inhibit glucose release from gluconeogenic substrates in either periportal or perivenous hepatocytes (37). Although the reason for this phenomenon is unclear, it is possible that components of the insulin signaling pathway necessary for repression of gluconeogenesis are disabled during the hepatocyte isolation procedure. Interestingly, these data imply that EGCG may act by a different mechanism than insulin, as discussed later.
FIG. 3. EGCG represses PEPCK and G6Pase gene expression in a PI3K-dependent manner. H4IIE cells were treated for 4 h with Dex/cAMP in the presence or absence of 10 nM insulin or increasing concentrations of EGCG. In experiments using kinase inhibitors, cells were pretreated with 20 μM LY 294002 or 25 μM U0126 for 30 min. Total RNA was isolated and used for primer-extension experiments to measure PEPCK or β-actin mRNA (panels A and B), as described under “Experimental Procedures.” PEPCK mRNA was normalized to β-actin mRNA and the response to Dex/cAMP was arbitrarily set at 100%. The data represent the average of six experiments ± S.E. (panel A) (**, p < 0.01, Student’s t test) or five experiments ± S.E. (panel B) (*, p < 0.05 Student’s t test). Ribonuclease protection assays were used to detect G6Pase and β-actin mRNA. The data shown in panel C is representative of five separate experiments (*, p < 0.05, Student’s t test).
RNA was isolated for primer extension analysis to measure PEPCK and $\beta$-actin (as a control) mRNA levels. EGCG, in a concentration-dependent manner, reduced PEPCK mRNA, as shown in Fig. 3 (panel A). Insulin reduces PEPCK gene expression by a PI3K-dependent mechanism, and the effect of insulin is blocked by the PI3K inhibitors wortmannin and LY 294002. MAPK is not involved, however, since MAPK/extracellular signal-regulated kinase kinase kinase inhibitors do not affect the regulation of PEPCK gene expression by insulin (22). H4IIE cells were treated with EGCG in the presence of LY 294002 or U0126, a MAPK/extracellular signal-regulated kinase kinase inhibitor, to determine whether PI3K or MAPK is involved in EGCG-mediated PEPCK gene repression. As observed with insulin, only LY 294002 reversed the effect of EGCG on PEPCK gene expression (Fig. 3, panel B), suggesting the involvement of PI3K, but not MAPK, in EGCG-mediated repression of the PEPCK gene. The G6Pase gene is hormonally regulated in a manner similar to that of the PEPCK gene, and insulin also represses this gene by a PI3K-dependent mechanism (38). The effect of EGCG on G6Pase expression was also examined using ribonuclease protection assays, with expression of the $\beta$-actin gene serving as a control (Fig. 3, panel C). Insulin and EGCG both repress expression of the G6Pase gene in a PI3K-dependent manner. These results suggest that EGCG mimics insulin action by repressing gluconeogenesis and the expression of genes that control hepatic gluconeogenesis.

**EGCG and Insulin Activate Similar Signaling Pathways—**

Insulin activates PI3K, PKB, and p70$^{s6k}$ in H4IIE cells (22). The effect of EGCG on these kinases was therefore measured using *in vitro* kinase assays (Table I). H4IIE cells were incubated with 10 nM insulin or 50 $\mu$g EGCG. Cell lysates were prepared and incubated with specific antibodies to immunoprecipitate IRS-1, PKB, or p70$^{s6k}$ for assay of PI3K, PKB, or p70$^{s6k}$, respectively. The fold stimulation relative to control is illustrated for each assay. The results represent three to four assays $\pm$ S.E. The effect of EGCG on these kinases was therefore measured using *in vitro* kinase assays (Table I). H4IIE cells were incubated with 10 nM insulin or 50 $\mu$g EGCG. Cell lysates were prepared and incubated with specific antibodies to immunoprecipitate IRS-1, PKB, or p70$^{s6k}$ for assay of PI3K, PKB, or p70$^{s6k}$, respectively. The fold stimulation relative to control is illustrated for each assay. The results represent three to four assays $\pm$ S.E.

### Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PI3K activity</th>
<th>PKB activity</th>
<th>p70$^{s6k}$ activity</th>
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</thead>
<tbody>
<tr>
<td>10 min</td>
<td>1.0 $\pm$ 0.0</td>
<td>1.0 $\pm$ 0.0</td>
<td>1.0 $\pm$ 0.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>96 $\pm$ 3.9</td>
<td>2.2 $\pm$ 0.7</td>
<td>2.2 $\pm$ 0.4</td>
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<tr>
<td>EGCG</td>
<td>4.3 $\pm$ 0.3</td>
<td>1.2 $\pm$ 0.2</td>
<td>1.2 $\pm$ 0.2</td>
</tr>
<tr>
<td>120 min</td>
<td>67 $\pm$ 0.4</td>
<td>3.1 $\pm$ 0.1</td>
<td>2.2 $\pm$ 0.2</td>
</tr>
<tr>
<td>Insulin</td>
<td>7.4 $\pm$ 1.2</td>
<td>1.4 $\pm$ 0.2</td>
<td>1.5 $\pm$ 0.5</td>
</tr>
<tr>
<td>EGCG</td>
<td>73 $\pm$ 14</td>
<td>3.6 $\pm$ 0.8</td>
<td>2.1 $\pm$ 0.1</td>
</tr>
<tr>
<td>240 min</td>
<td>13 $\pm$ 1.7</td>
<td>1.3 $\pm$ 0.1</td>
<td>1.6 $\pm$ 0.1*</td>
</tr>
</tbody>
</table>

*p < 0.05, Student’s *t* test.*

Insulin increases tyrosine phosphorylation on IR-$\beta$, IRS-1, and IRS-2. IR-$\beta$ and IRS-1 were therefore immunoprecipitated and analyzed for tyrosine phosphorylation using phospho-tyrosine-specific antibodies to determine whether EGCG affects the phosphorylation of these proteins in H4IIE cells. These cells do not express sufficient amounts of IRS-2 for analysis. Insulin and EGCG both increase the tyrosine phosphorylation of IR-$\beta$ and IRS-1 (Fig. 4, panel B). Insulin increased the tyrosine phosphorylation of both proteins within 30 min, as expected. EGCG also increased the levels of tyrosine phosphorylation within 30 min, but further increases were noted up to 3 h. The extent of tyrosine phosphorylation of these proteins elicited by EGCG was not as robust as that observed with insulin. However, EGCG does promote the association of active PI3K with IRS-1, as shown in Table I.

The effect of EGCG on tyrosine phosphorylation of the IGF-1 receptor (IGF-1R) was also examined (Fig. 4, panel C). H4IIE cells express low levels of IGF-1R, so Hep G2 cells were used for this experiment. Hep G2 cells were incubated with IGF-1 or EGCG, and the $\beta$-subunit of the IGF-1R was immunoprecipitated for analysis with the phospho-tyrosine-specific antibody. As observed with the insulin receptor, EGCG caused a small delayed increase in tyrosine phosphorylation of the IGF-1R $\beta$-subunit.

**EGCG Has Pro-oxidant Activity in Hepatoma Cells—**ROS have been implicated in the regulation of protein kinase cascades and in the inhibition of PTPs (39, 40). It is therefore possible that pro-oxidative activity of EGCG in hepatoma cells could explain the increased levels of tyrosine-phosphorylated proteins observed in these cells. H4IIE cells were incubated with DCFH to test whether EGCG increases ROS production. ROS produced in cells causes oxidation of DCFH, yielding the fluorescent product DCF (36). H4IIE cells were treated in the presence or absence of EGCG, and DCF fluorescence was measured (Fig. 5). A punctate pattern of fluorescence was seen after EGCG treatment, most of which was localized in the perinuclear region. This suggests that EGCG has pro-oxidant activity in hepatoma cells. Insulin had no effect on DCF fluorescence (data not shown). The increase in DCF fluorescence was dose-dependent, because approximately half the fluorescence was measured when cells were treated with 25 $\mu$g EGCG compared to untreated cells.
Regulation of Signaling Pathways by EGCG

with 50 μM EGCG (56.2 ± 7.0 compared with 109.4 ± 48, respectively), when assessed by a spectrofluorometer (excitation, 500 nm; emission, 530 nm). It was difficult to accurately measure the fluorescence at lower EGCG concentrations. The EGCG-mediated increase in DCF fluorescence was abolished by co-treating the cells with N-acetylcysteine (NAC), a glutathione precursor and scavenger of ROS (Fig. 5). Superoxide dismutase (SOD), a scavenger of superoxide anions, also decreased the number of cells that fluoresced. These results suggest that EGCG increases ROS production in H4IIE cells. Despite the rise in ROS, treatment of H4IIE cells with up to 1 mM EGCG had no adverse effects on cell viability as assessed by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assays (data not shown).

NAC and SOD Reverse the Effect of EGCG on Tyrosine Phosphorylation and PEPCK/G6Pase Gene Expression—Because ROS production seems increased after treatment of H4IIE cells with EGCG, the effect of NAC and SOD on tyrosine phosphorylation of H4IIE cellular proteins was examined. H4IIE cells were treated with NAC or SOD for 30 min before treatment with insulin or EGCG for 2 h. Cells were harvested, and cell lysates prepared as described in Fig. 4, panel A. Both NAC and SOD completely reversed the effect of EGCG on protein-tyrosine phosphorylation (Fig. 6). SOD had no effect on insulin-mediated protein-tyrosine phosphorylation. However, NAC partially reversed the tyrosine phosphorylation of proteins around 85 kDa but did not affect the tyrosine phosphorylation of larger proteins (around 150–200 kDa) (Fig. 6, panel A). The effect of NAC and SOD on PEPCK and G6Pase gene expression was also examined. As expected, NAC and SOD completely reversed EGCG-mediated PEPCK and G6Pase gene repression. NAC partially inhibited the effect on insulin-mediated repression of the PEPCK gene, but not the G6Pase gene (Fig. 6, panels B and C). These results show that EGCG regulates tyrosine phosphorylation and gene expression by a redox-dependent mechanism and provides additional evidence that the PEPCK and G6Pase genes are regulated by multiple signaling pathways.

DISCUSSION

Although the Western diet is thought to contribute to an increased lifetime risk of certain diseases, such as cancer and diabetes, plant-based diets offer protective effects (41–43). Tea consumption, especially green tea, is associated with a lower incidence of human cancer (6). EGCG, the main polyphenolic constituent of green tea, may prevent carcinogenesis by several
different mechanisms, including inhibition of angiogenesis, impairment of cell cycle progression, induction of glutathione S-transferase, and decreased production of ROS (6, 41–45).

Several reports have suggested that EGCG and related compounds possess antidiabetic activity and EGCG significantly decreases blood glucose when injected into lean and obese Zucker rats (8, 10, 46). Our results reveal that EGCG is insulinomimetic in that it lowers glucose production in H4IIE cells and decreases the expression of genes that control gluconeogenesis, such as the PEPCK and G6Pase genes. Also, EGCG activates the same kinases as insulin and promotes the phosphorylation of insulin signaling proteins, such as IRS-1 and IR-β. Interestingly, EGCG has similar effects in primary hepatocytes and hepatoma cells in that it increases the level of tyrosine-phosphorylated proteins, including the insulin receptor, and it represses PEPCK gene expression (data not shown).

The effects of EGCG are reversed by NAC and SOD, whereas those of insulin are mostly unaffected, suggesting that the former acts by a different mechanism. In most cell types, EGCG is an antioxidant. However, in hepatoma cells, EGCG is a pro-oxidant. This is not completely unexpected because other compounds, such as ascorbate, can act either as an antioxidant or pro-oxidant, depending on the cellular environment (47). Curcumin, a phytochemical responsible for the color of turmeric, has antioxidant activity in many different cell types but displays pro-oxidant qualities in the presence of transition metals, such as copper, which exist in the kidney and liver at relatively high concentrations (48).

The data presented here suggest that EGCG regulates protein-tyrosine phosphorylation by modulating the redox state of...
the cell. One possible mechanism for the observed actions of EGCG in hepatoma cells is the inhibition of PTPs, which contain an oxidizable cysteine in their active site (39, 49). It is possible that EGCG causes oxidation of this cysteine residue in redox-sensitive phosphatases, and NAC and SOD reverse this effect. Several PTPs, including PTP-1B and leukocyte antigen-related phosphatase, dephosphorylate the insulin receptor and IRS-1, making these phosphatases candidates for modification by ROS produced in response to EGCG (50–52). It is noteworthy that disruption of the PTP-1B gene in mice leads to symptoms similar to those observed in Zucker rats injected with EGCG, such as decreased obesity and blood glucose levels and increased insulin sensitivity (8, 53, 54). We are currently testing the effect of EGCG on purified PTPs to test this idea.

This study demonstrates that EGCG causes many of the same cellular effects as insulin, including repression of glucose production and PEPCk and G6Pase gene expression. EGCG, however, seems to exert these effects by modulation of the redox state of the cell. Thus, EGCG analogs or other novel phytochemicals may be identified that have insulin-like effects. Further experiments directed at determining the mechanisms of EGCG action may lead to the identification of molecular targets for the generation of therapeutic agents useful in the treatment of diabetes.

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